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WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12N 15/00, 9/02, 15/62 A01K 67/027, C12N 1/19, 9/10 C12N 15/12, 15/53	A1	(11) International Publication Number: WO 92/14817
		(43) International Publication Date: 3 September 1992 (03.09.92)

(21) International Application Number: PCT/GB92/00274

(22) International Filing Date: 17 February 1992 (17.02.92)

(30) Priority data:
9103314.2   16 February 1991 (16.02.91)   GB

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(81) Designated States: AT (European patent), BE (European patent), CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), MC (European patent), NL (European patent), SE (European patent), US.

Published
With international search report.

(54) Title: IN VIVO ASSAY SYSTEMS FOR METABOLIC ROUTES

(57) Abstract

A cellular organism useful in an assay for determining the metabolism of a compound, the organism comprising in the genome of its cell or at least one of its cells a coding sequence for expressing a polypeptide having the function of a naturally-occurring protein which is involved in the alteration of the metabolism, mutagenicity or toxicity of a compound under the regulatory control of a suitable promoter, the combination of the coding sequence and the promoter not normally being found in the said cell of the said organism. Preferably, the protein is a P450 cytochrome-dependent enzyme. The organism may be yeast (in which case a mammalian NADPH:cytochrome P450 reductase or a hybrid yeast/mammalian P450 reductase can usefully be encoded as well), a rodent (in which case expression in the skin using a keratin promoter is preferred, optionally with co-expression of a glutathione S-transferase) or a *Drosophila* fly.

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IN VIVO ASSAY SYSTEMS FOR METABOLIC ROUTES

The present invention relates to assay systems for
determining the metabolic fate of a compound in mammals,
particularly in humans. It also relates to transgenic
organisms *per se*, useful in these and other contexts.

The manner in which exposure to chemicals in the
environment influences the incidence of human disease is a
central issue at the present time. The rate of metabolism
and disposition of such compounds is of central importance
in determining our response to such compounds. These
reactions are catalyzed by a broad range of proteins
generally termed drug metabolizing enzymes. Individuality
in the expression of these proteins may be a critical
determinant in susceptibility to diseases such as cancer.
On this premise it is of obvious importance to clearly
identify the metabolic routes of chemical toxin and
carcinogen disposition. A limitation of almost all studies
to date, on this theme, has been the lack of *in vivo* models to
evaluate the role of specific enzymes in toxicological
response.

The cytochrome P450-dependent monooxygenases play a
critical role in the metabolism and disposition of foreign
chemicals.   These enzymes oxidise potentially toxic
lipophilic compounds to products which are subsequently
conjugated and excreted as water soluble metabolites.
Ironically, the enzymes can convert non-toxic compounds to
highly reactive chemical intermediates such as epoxides
which react with DNA, RNA or protein producing cytotoxic,
genotoxic and carcinogenic effects (Wolf, 1986). In view
of its critical function in detoxification the cytochrome
P450 monooxygenase system has diverged into several
multigene families encoding proteins with distinct but
overlapping substrate specificities. Because the response
of an individual to a particular drug, toxin or carcinogen

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could be the product of several different enzyme activities
it is extremely difficult to assess the relative
contribution of each enzyme in determining the overall
effect. The problem is further compounded by there being
5   polymorphisms in the genes involved which, in turn, alter
the susceptibilities of individuals to particular
chemicals. Variation between individuals also depends upon
the previous history of exposure to chemicals or drugs
because a number of the genes are inducible by a variety of
10 different chemical agents (Nebert and Gonzalez, 1987;
Adesnik and Atchison, 1986). In order to unravel this
bewildering complexity of factors a versatile *in vivo* model is
required.

15 The Somatic Mutation and Recombination Test (SMART) (Graf
et al, 1984; Szabad *et al*, 1983; Szabad, 1986; Frölich and
Würgler, 1989) involves exposure of *Drosophila* larvae,
heterozygous for different recessive cuticle markers, to a
potential mutagen. Clones of marked cells in the imaginal
20 discs are induced by a number of genotoxic effects:
somatic mutation, chromosomal rearrangements or
nondisjunction. These events are subsequently manifest as
mosaics in the differentiated structures of the adult.
This is a single generation test which involves exposing
25 larvae to the potential mutagen.

Cytochrome P450-dependent monooxygenases (P450s) are a
supergene family of enzymes that catalyse the oxidation of
lipophilic chemicals through the insertion of one atom of
30 molecular oxygen into the substrate. They are involved in
the metabolism of a vast range of xenobiotic compounds, and
in particular with the clearance of almost all
pharmaceutical drugs. The P450 system is polymorphic in
man, and genetic differences in the P450-mediated
35 metabolism of a wide variety of drugs have been clearly
demonstrated. The best example of this is the
debrisoquine/sparteine polymorphism. Up to 10% of the

Caucasian population exhibit the poor metaboliser (PM) phenotype. This is characterised by a significantly reduced ability to metabolise the prototype drug debrisoquine to 4-hydroxydebrisoquine, the metabolism being
5   10-200 times less than in extensive metabolisers (EMs). The PM phenotype is inherited as an autosomal recessive trait, and up to 54% of people are carriers of a mutant allele(s). The PM phenotype leads to impaired clearance of over twenty other commonly prescribed drugs including (in
10 the cardiovascular area) metoprolol, timolol, propranolol, perhexilene, N-propylamaline, propafenone, encainide, flecainide and mexiletine, (in the psychiatric area) amitriptyline, imipramine, desipramine, nortriptyline, clomipramine, thioridazine, perphenazine, amiflamine and
15 tomoxitene and (in other areas) codeine, methoxyphenamine, cyclophosphamide and phenformin and possibly also chlorpropamine, melatonin and MPTP and may result in serious adverse side effects upon their administration. It is thought that almost all carcinogenic agents are
20 converted by P450 enzymes into their ultimate carcinogenic form.

One aspect of the present invention provides a cellular organism useful in an assay for determining the metabolism
25 of a compound, the assay comprising a transgenic cellular organism comprising in the genome of its cell or at least one of its cells a coding sequence for expressing a polypeptide having the function of a naturally-occurring protein which is involved in the alteration of the
30 mutagenicity or toxicity of a compound under the regulatory control of a suitable promoter, the combination of the coding sequence and the promoter not normally being found in the said cell of the said organism.

35 Preferably, the said naturally-occurring protein is one involved in determining the susceptibility of a cell to a toxic or mutagenic chemical for example mdr (multiple drug

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resistance) transport protein, a glutathione-S-transferase, a UDP-glucuronosyl transferase, epoxide hydrolase or an enzyme from the superfamily of P450 cytochrome dependent enzymes. In this specification, for convenience, the terms

5   "P450", "P450 enzyme" and "P450 activity" are used to refer to enzymes of the said superfamily and their activity.

There is one microsomal/mitomondrial form and several cytosolic forms of GST (EC 2.5.1.18). The GST isozymes

10   exhibit overlapping substrate specificities, including alkyl and aryl halides, aromatic amines, lipid hydroperoxides, α,β-unsaturated ketones, quinones and epoxides.

15   The P450 gene may be any of those identified in Nebert *et al* (1987) *DNA* 6, 1-11, Nebert *et al* (1989) *DNA* 8, 1-13 or any subsequent update.

Present knowledge indicates that each P450 gene usually

20   produces a single protein (the enzyme). To date, there appear to be several exceptions to this rule where "functional" alternative splicing might occur, ie differential processing of the P450 transcript such that entire exons or portions of exons are exchanged in order to

25   produce an enzyme with a new catalytic activity.

There are presently 141 P450 genes and six pseudogenes that have been described in 24 eukaryotes (including ten mammalian and two plant species) and in four prokaryotes

30   (Table B). Of the 26 gene families so far described, ten exist in all mammals. These ten families comprise 19 subfamilies, or clusters of genes, of which 15 and 11 have been mapped in the human and murine genome, respectively (Table A).

TABLE A: CHROMOSOMAL AND SUBCHROMOSOMAL LOCALISATION OF CYP GENES

P450 cluster or gene	Chromosomal location	References HUMAN
CYP1	15q22-qter (near MPI)	
CYP2A	19q13.1-13.2	Miles et al (1990a)
CYP2B	19q12-q13.2	Miles et al (1988)
		Yamano et al (1989)
CYP2C	10q24.1-24.3	Shephard et al (1989)
CYP2D	22q11.2-qter	
CYP2E	10	
CYP2F	19	Nhamburo et al (1990)
CYP2G		
CYP3A	7q21.3-q22	
CYP3B		
CYP4A	1	O.W. McBride and J.P. Hardwick in preparation
CYP4B	1p12-p34	Nhamburo et al (1989)
CYP7		
CYP11A	15	
CYP11B	8q21	
CYP17	10	
CYP19	15	

CYP21	6p (within HLA)	6
CYP26	2q33-qter	J.J. Cali, D.W. Russell, U. Francke *et al* in preparation

MOUSE

Cyp1	Mid-9 (near Mpi-1)	
Cyp2a	7 (near Gpi-1)	Matsunage, T. *et al* (1990)
		Miles *et al* (1990b)
		Burkhart *et al* (1990)
Cyp2b	Proximal 7 (Coh)	Miles *et al* (1990b)
Cyp2c	19	
Cyp2d	15	Wong *et al* (1989)
Cyp2e	7	
Cyp2f	7	Nhamburo *et al* (1990)
Cyp2g		
Cyp3a	5	
Cyp3b		
Cyp4a	4	Kimura, S. *et al* (1989b)
Cyp4b		
Cyp7		
Cyp11a	9	Youngblood *et al* (1989)

Cyp11b
Cyp-17
Cyp-19
Cyp-21　　　　17 (within H-2)
5　　Cyp-26　　　　1　　　　J.J. Cali, D.W. Russell, U. Francke *et al*, in
preparation

10　References are not included in this Table if the work was cited in the Nebert *et al* (1989) update.

TABLE B:　UPDATE OF ALL CYP GENES AND THEIR PRODUCTS

Gene symbol	Trivial name	Species	References
CYP1A1	c, βNF-B	rat	
	P_1	mouse	
	P_1, c, form 6	human	
	form 6	rabbit	
	IA1	trout	
	Dah1	dog	
	MKah1	monkey	
CYP1A2	P-448, d, HCB	rat	
	P_3, P_2	mouse	
	P_3, d, form 4	human	

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	LM$_4$	rabbit	
	MC4	hamster	
	Dah2	dog	Lai and Chiang (1990)
5	CYP2A1	a1,a,3,UT-F	rat	Matsunaga, T. *et al* (1990)
	CYP2A2	a2,RLM2	rat	Matsunaga, T. *et al* (1990)
	CYP2A3	.a3	rat	Kimura, S. *et al* (1989c)
	Cyp2a-4	15αoh-1	mouse	Lindberg *et al* (1989)
				Burkhart *et al* (1990)
10	Cyp2a-5	15αoh-2	mouse	Lindberg *et al* (1989)
				Burkhart *et al* (1990)
	CYP2A6	11A3,P450(1)	human	Yamano *et al* (1989a, 1990)
				Miles *et al* (1990a)
	CYP2A7	AFB	human	Fukuhara *et al* (1989)
15	CYP2A8	MC1	hamster	Lai and Chiang (1990)
	CYP2B1	b,PB-4,PB-B	rat	
	CYP2B2	e,PB-5,PB-D	rat	
	CYP2B3	IIB3	rat	
20	CYP2B4	LM2,B0,b15,B1,b46	rabbit	
	CYP2B4P	(pseudogene)	rabbit	
	CYP2B5	b52,B2,HP1	rabbit	

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CYP2B6	LM2	human	
CYP2B7	IIB1	human	Yamano et al (1989b)
CYP2B8	IIB2 (pseudogene?)	human	Yamano et al (1989b)
Cyp2b-9	pf26	mouse	
Cyp2b-10	pf3/46	mouse	
CYP2B11	hIIB	human	Miles et al (1988)
CYP2B12	gene IV	rat	Giachelli et al (1989)
CYP2B13	IIB	dog	Graves et al (1990)
CYP2C1	PBc1	rabbit	
CYP2C2	PBc2,K,pHP2	rabbit	
CYP2C3	PBc3,3b	rabbit	Zhao et al (1990)
CYP2C4	1-88,PBc4	rabbit	Chan and Kemper (1990)
CYP2C5	form 1	rabbit	Zhao et al (1990)
CYP2C6	PB1,K,PB-C	rat	
CYP2C6P	(pseudogene)	rat	
CYP2C7	f,pTF1	rat	Kimura, H. et al (1989)
CYP2C8	form 1,IIC2,mp-12, mp-20	human	
CYP2C9	IIC1,mp-4	human	
CYP2C10	mp,mp-8	human	Romkes et al (1990)
CYP2C11	h,M-1,16α,2c,UT-A	rat	

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Gene	Clone	Species	Reference
CYP2C12	i,15β,2d,UT-1	rat	Zaphiropoulos et al (1990b)
CYP2C13	+g	rat	McClellan-Green et al (1989)
			Zaphiropoulos et al (1990a)
	-g	rat	Yeowell et al (1990)
CYP2C14	pHP3	rabbit	
CYP2C15	b32-3	rabbit	
CYP2C16	IIC16	rabbit	Hassett and Omjecinski (1990)
?CYP2C17	pB8	human	Shephard et al (1989)
?CYP2C18	254c	human	Romkes et al (1990)
?CYP2C19	29c,6b	human	
?CYP2C20	11a	human	
?CYP2C21	MKmp13	monkey	M. Komori, pers. commun.
?CYP2C22	DM 1-1	dog	M. Komori, pers. commun.
CYP2D1	db1	rat	Matsunaga, E. et al (1989)
CYP2D2	db2	rat	Matsunaga, E. et al (1989, 1990)
CYP2D3	db3	rat	Matsunaga, E. et al (1989, 1990)
CYP2D4	db4	rat	Matsunaga, E. et al (1989, 1990)
CYP2D5	db5,CMF1b	rat	Matsunaga, E. et al (1989, 1990)
CYP2D6	db1	human	Ishida et al (1989)
CYP2D7	IID7	human	Kimura, S. et al (1989d)

CYP2D8	IID8	human	Kimura, S. *et al* (1989d)
Cyp2d-9	16α,ca	mouse	Wong *et al* (1989)
Cyp2d-11	cc	mouse	Wong *et al* (1989)
Cyp2d-12	cd	mouse	
Cyp2d-13	ce	mouse	
CYP2E1	j	human	
	j	rat	
	3a	rabbit	
	MKj1	monkey	
CYP2E2	IIE2	rabbit	
CYP2F1	IIF1	human	Nhamburo *et al* (1990)
CYP2G1	olf1	rat	Nef *et al* (1989, 1990)
	NMb	rabbit	Ding *et al* (1990)
CYP2H1	pCHP3	chicken	Hansen and May (1989)
CYP2H2	pCHP7	chicken	Hansen and May (1989)
CYP3A1	pcn1	rat	
CYP3A2	pcn2	rat	
CYP3A3	HLp	human	

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CYP3A4	nf-25,hPCN1,nf-10	human	
CYP3A5	hPCN3	human	Aoyama *et al* (1989)
CYP3A6	3c	rabbit	
CYP3A7	HLp2	human	Schuetz *et al* (1989)
CYP3A8	HFL33	human	Komori *et al* (1989a, 1989b)
CYP3A9	MKnf2	monkey	
CYP3B1	olf2	rat	
CYP4A1	LA 1	rat	Kimura, S. *et al* (1989a)
CYP4A2	IVA2	rat	Kimura, S. *et al* (1989a)
CYP4A3	IVA3	rat	Kimura, S. *et al* (1989b)
CYP4A4	p-2	rabbit	
CYP4A5	KDB3	rabbit	Johnson *et al* (1990)
CYP4A6	R9, KDA6,ka-1	rabbit	Yokotani *et al* (1989)
			Johnson *et al* (1990)
CYP4A7	R4, KDB18,ka-2	rabbit	Yokotani *et al* (1989)
			Johnson *et al* (1990)
CYP4A8	p-2-like	human	Yokotani *et al* (1990)
CYP4A9	PP1	rat	Stromstedt *et al* (1990)

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CYP4B1	lung P450	human	Nhamburo *et al* (1989)
	form 5	rabbit	Gasser and Philpot (1989)
	form 5	rat	Gasser and Philpot (1989)
CYP4C1	P-450	cockroach	Y.H. Lee, L.L. Keeley, J.Y. Bradfield, in preparation
CYP6	VIA1	house fly	Feyereisen, R. *et al* (1989)
CYP7	7α	rat	Noshiro *et al* (1989)
			Jelinek *et al* (1990)
			Li *et al* (1990)
		human	Noshiro and Okuda (1990)
		rabbit	
		cow	
		baboon	
CYP10	P-450	pond snail	
CYP11A1	scc	human	
		cow	Ahlgren *et al* (1990)
		pig	Mulheron *et al* (1989)

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CYP11B1   11β   rat   Oonk *et al* (1989)

5   human   Mornet *et al* (1989)
                     cow   Kirita *et al* (1988)
                               Hashimoto *et al* (1989)
                     rat   Nonaka *et al* (1989)
                               Naomichi *et al* (1990)
                               Imai *et al* (1990)
                               Matsukawa *et al* (1990)

CYP11B2   (pseudogene?)   human   Mornet *et al* (1989)

10 **CYP17**   17α   human   Bhasker *et al* (1989)

                     cow
                     pig
                     chicken
15   rat   Namiki *et al* (1988)
                               Fevold *et al* (1989)
                               Mellon and Vaisse (1989)

CYP19   arom   human   Means *et al* (1989)
20   Harada *et al* (1990)

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Gene	Code	Species	Reference
CYP21A1	c21	rat	Lephart *et al* (1990)
		cow	Hickey *et al* (1990)
CYP21A1P	c21A	mouse	
CYP21A2	c21	pig	
Cyp21a-2p	(pseudogene c21A)	human	
	c21B	human	
	(pseudogene c21B)	mouse	
CYP26	26-ohp	rabbit	Andersson *et al* (1989)
		rat	Usui *et al* (1990)
		human	Su *et al* (1990)
CYP51	14DM	*S. cerevisiae*	
		C. tropicalis	
		C. albicans	Lai and Kirsch (1989)
CYP52A1	alk1	*C. tropicalis*	Sanglard and Loper (1989)
CYP52A2	alk2	*C tropicalis*	Sanglard and Fiechter (1989)

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CYP52A3	Cm-1	*C. maltosa*	Schunck *et al* (1989)
CYP52A4	Cm-2	*C. maltosa*	
CYP53	bphA	*Asp. niger*	R. van Gorcom, pers. commun.
CYP54	C1-1	*N. crassa*	Attar *et al* (1989)
CYP55	dNIR	*E. oxysporum*	Kizawa *et al* (1990)
CYP56	DIT2	*S. cerevisiae*	Briza *et al* (1990)
CYP71		Avocado	Bozak *et al* (1990)
CYP101	cam	*Ps. putida*	
CYP102	BM-3	*B. megaterium*	Ruettinger *et al* (1989)
CYP103	pinF1	*Agr. t'faciens*	Kanemoto *et al* (1989)
CYP104	pinF2	*Agr. t'faciens*	Kanemoto *et al* (1989)
CYP105A1	SU1	*Str. griseolus*	Omer *et al* (1990)

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CYP105B1	SU2	*Str. griseolus*	Omer *et al* (1990)
CYP105C1	choP	*Str. sp.*	Horii *et al* (1990)
CYP106	BM-1	*B. megaterium*	He *et al* (1989)

The References are listed in Nebert *et al* (1991) *DNA & Cell Biol.* **10**(1), 1-14. References are not included in this Table if the work was cited in the Nebert *et al* (1989) update. Okada *et al* (1989) reported a plant oxidase cDNA sequence exhibiting similarities to the NH_2 terminus of the mouse Cyp1a-1 protein; since this gene does not have the cysteinyl-containing heme-binding site in the COOH terminus, it does not qualify as a member of the P450 gene superfamily.

In one embodiment, the organism is a eukaryote, such as a
Drosophila (eg *D. melanogaster*), a rodent (eg a rat or a mouse)
or a yeast (eg *Saccharomyces cerevisiae*). In a multi-cellular
organism, the said promoter preferably provides for
5   expression in only some cell types of the organism, for
example the fat body (the insect equivalent of the liver)
of the third instar *Drosophila* larva (eg using the *Drosophila*
LSP1α gene promoter) or the skin of a rodent (eg using the
bovine keratin VI promoter). However, in the case of
10   short-lived putative carcinogens, it may be appropriate to
provide for expression in most or all tissues of the host.
In *Drosophila*, for example, this can be achieved with the
Hsp70 promoter. When the promoter is such as to cause
expression of the P450 enzyme in only some cells of the
15   organism or in only some developmental stages of the
organism, then clearly the organism is used in the assay
methods of the invention such that the test compound is
applied directly or indirectly to the cells or at the
relevant developmental stage.

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At least in lower eukaryotes such as yeast, and also in
prokaryotes such as *Salmonella* spp., it may be found that the
natural mammalian P450 enzyme does not function adequately.
Hybrid enzymes can be constructed to provide a region
25   having mammalian (eg human) P450-type enzymatic specificity
and a region having the requisite lower eukaryote or
prokaryote function. For example, the polypeptide can be
so encoded as to have a human P450 enzyme region and a
region from the yeast P450 enzyme which will bind yeast
30   membranes and thereby increase the enzymatic activity of
the polypeptide in yeast. Alternatively, the portion of
the mammalian P450 enzyme which does not allow adequate
expression in the lower eukaryote or prokaryote (for
example the hydrophobic amino terminal amino acids) may
35   simply be deleted.

It is necessary for the mammalian P450 enzyme to be
expressed with the co-enzyme NADPH:cytochrome P450
reductase. In some hosts, for example lower eukaryotes and
prokaryotes, the endogenous reductase enzyme may not be
5 present or may not form a functional relationship with the
mammalian P450 monooxygenase. One solution is to co-
transform the organism with a gene for a suitable (eg
mammalian) co-enzyme, another is to express a hybrid,
fusion protein comprising the enzymatically active portions
10 of the P450 enzyme and mammalian reductase and a third is
to express a similar fusion protein but using the host's
reductase, for example bacterial or yeast reductase.

Thus, the fusion protein of the invention may comprise a
15 first region providing the activity of a naturally-
occurring protein from a first organism which protein is
involved in the alteration of the mutagenicity or toxicity
of a compound and a second region adapted to bind to cell
membranes, the second region being homologous to a cell-
20 binding region of a protein in an organism other than the
first organism.

Preferably, the first organism is a mammal and the said
naturally-occurring protein is the mdr transport protein,
25 a glutathione S-transferase, P450 reductase or an enzyme of
the superfamily of P450 cytochrome dependent enzymes.

Suitably, the second region is adapted to bind to yeast
cell membranes.
30
Advantageously, the fusion protein comprises the sequence
$H_2N-R^1-R^2-R^3-COOH$ wherein R^1 is homologous to the n N-terminal
amino acids of *Saccharomyces cerevisiae* P450 reductase where n is
10-30, R^2 is homologous to the n' N-terminal amino acids of
35 human P450 reductase where n' is 10-56, and R^3 provides the
enzymatic function of human P450 reductase.

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By "homologous" we mean that the two sequences being compared have at least 30% identity of amino acids, preferably at least 50%, 75%, 90%, 95% or 99% identity. Preferably, R^3 comprises the soluble portion of mammalian
5   P450 reductase or a homologous amino acid sequence, for example the 56-678 region.

The main purpose of attaching the transgenic product to the host's cell membranes is to bring the P450 enzyme and the
10 P450 reductase into mutual proximity. The same end effect may be achieved without attachment to membranes if the two enzymes are expressed as a fusion protein. Thus, a further molecule of the invention comprises a first region having the enzymatic function of a mammalian P450 enzyme and a
15 second region having the enzymatic function of a P450 reductase, preferably a mammalian P450 reductase.

The said enzymatic function of the P450 enzyme is to oxidase substrates as described above and it will normally
20 bind haem (present in yeast) to do this. The enzymatic function of the reductase is to channel electrons to the P450 enzyme and it will normally bind one molecule each of flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) to do this. Thus, the said first region will
25 normally (a) bind haem, and, when haem is bound, (b) bind a P450 enzyme substrate such as one or more of those listed above, (c) react with carbon monoxide to give the characteristic 450 nm optical band and (d) oxidise the said substrate. It is typically 50-60 kDa in mclecular weight.
30 The second region will normally have NADPH-dependent cytochrome c reductase activity and is typically 60-70 kDa in molecular weight. The sequence of each region may be derived from the corresponding sequences of P450 and reductase, omitting any hydrophobic terminal region which,
35 in the native molecule, serves to bind membranes.

Non-enzymatically active linker and terminal extension

regions may be included if desired. The said first region
may be N-terminal or C-terminal to the said second region.

Particular fusion proteins include the following:

	First Region	Second Region
(i)	full length mammalian (preferably human) P450 enzyme	full length mammalian (preferably human) reductase
(ii)	full length mammalian (preferably human) P450 enzyme	full length *Bacillus megaterium* reductase
(iii)	full length mammalian (preferably human) P450 enzyme	mammalian (preferably human) reductase, less hydrophobic domain
(iv)	mammalian (preferably human) P450 enzyme, less hydrophobic domain	full length mammalian (preferably human) reductase
(v)	mammalian (preferably human) P450 enzyme, less hydrophobic domain	full length *Bacillus megaterium* reductase
(vi)	mammalian (preferably human) P450 enzyme, less hydrophobic domain	full length *Bacillus megaterium* reductase
(vii)	*Bacillus megaterium* P450 domain	full length mammalian (preferably human) reductase
(viii)	*Bacillus megaterium* P450 domain	full length *Bacillus megaterium* reductase
(ix)	*Bacillus megaterium* P450 domain	full length *Bacillus megaterium* reductase

In each case, the first region is preferably N-terminal to
the second region.

In the truncated mammalian reductase (rat or human),
regions of the hydrophobic membrane anchor will be removed
ie amino acids 1-56. For the truncated P450s, the

hydrophobic tail will be removed between amino acids 1-50.

. A linker may be included between the first and second
regions. The size of the linker region may be in the order
5   of 6-20 amino acids. The *B. megaterium* linker sequence(s) or
a similar hydrophilic linker may be used. (The linker
region of the *B. megaterium* fusion contains approximately 20
amino acids of which 8 of these are charged and 7 are
hydrophilic). The linker preferably incorporates a
10 multiple cloning site to facilitate a variety of different
P450 cDNA sequences. The *Bacillus megaterium* P450/reductase
molecule is described in Li *et al* (1991), Narhi *et al* (1987)
and Ruettinger *et al* (1989). These fusion compounds may be
expressed in any suitable host (eg *Salmonella*) if they are
15 soluble.

It may be advantageous for the cells in which the P450
activity is expressed to have the glutathione S-transferase
(GST) activity deleted. GST is a general detoxifying enzyme
20 and it may be informative to study the effect of the P450
activity on the compound without GST interfering with the
compound's P450 metabolism. Alternatively, especially in
non-mammalian hosts expressing mammalian P450 activity, it
may be advantageous to co-express mammalian GST precisely
25 to mimic the normal human situation. When doing this, if
the host tissue has a significant level of endogenous GST,
it may be advantageous to delete the host's GST gene(s) so
that only the transgenic GST is expressed. The *Drosophila GST*
genes have been described in Toung *et al* (1990) and it is
30 possible to select for P element insertions which prevent
GST gene expression using known methods, for example those
of Ballinger & Benzer (1989) or Kaiser & Goodwin (1990).
Alternatively, or as well, the level of transgenic protein
(for example GST) may be increased (in absolute terms or in
35 relation to endogenous protein, if present) by inserting
more than one copy of the transgene.

Transgenic organisms expressing useful levels of mammalian P450 enzyme activity as indicated above are novel and form a further aspect of the invention, whether or not they are used in the assay systems of the first aspect of the

5   invention. Preferably, the organism is a higher eukaryote or a prokaryote. Although attempts have previously been made to express mammalian P450 enzymes in yeast, these have only produced low levels of P450 activity. By deleting the hydrophobic tail of the enzyme and optionally replacing it

10   with a hydrophobic tail from a host (yeast) enzyme, useful levels of mammalian P450 activity can be obtained. "Higher eukaryotes" in this specification means multicellular eukaryotes, such as insects, fish, birds and mammals.

15   The invention also encompasses methods of making the transgenic organisms by transfection or transformation with the polypeptide-encoding sequence and an appropriate promoter; isolated constructs comprising the polypeptide-encoding sequence and a promoter which is (i) heterologous

20   to the coding sequences (ie not naturally found in regulatory association therewith) and (ii) effective in a higher eukaryote; coding sequences for the said hybrid enzymes; and the hybrid enzymes themselves.

25   A further aspect of the invention provides a method of assessing the toxicity of a compound, the method comprising exposing an organism as described above to the compound and determining either the metabolism of the compound or the effect of the compound on the organism, its development or

30   its progeny.

The organisms of the invention may be used in conjunction with the toxicity screening methods of, for example, EP 289 121, US 4 753 874, WO 89/05864, WO 89/09272, EP 370 813,

35   Oda *et al* (1985 *Mutat. Res.* **147**, 219-229) or Hall *et al* (1983 *J. Mol. App. Genet.* **2**, 101-109). Thus, the host organism of those various systems, particularly where it is prokaryotic or

lower eukaryotic, can be improved by engineering it to express a human P450 enzyme or other protein in accordance with the invention, so that the assay gives a better indication of what really happens in a human exposed to the

5   compound being screened.

The transgenic organisms, particularly prokaryotes such as *E. coli* and *Salmonella spp.*, may also be used to degrade environmentally harmful compounds to less harmful compounds

10   and may therefore be used to detoxify industrial waste and spillages of, for example, oil. Such organisms may contain transgenes with other compound-degrading capabilities or may be used in conjunction with other organisms to provide a broad spectrum of degradative capabilities.

15

Aspects of the invention will now be illustrated by way of example (all references being incorporated herein by reference) and with reference to the accompanying drawings in which the figure legends are as follows:

20

Figure 1. Structure of the transgene constructs introduced into the *Drosophila* germline.

Figure 2. Breeding scheme for selection of transformants.

25   SM1, Cy: second chromosome balancer carrying dominant Curly wings marker.   TM3, Sb ry^{RK}:   third chromosome balancer with dominant Stubble bristle marker has a mutant rosy gene. α^F: LSP1alpha fast electrophoretic variant. β^0:   LSP1beta null allele. Γ^0 2' ry: LSP1gamma deficiency,

30   LSP2 slow electrophoretic variant, rosy.

Figure 3. Genomic Southern of DNA from each of the two *alacZ* transformed lines and the two *αCYP2B1* lines.   The DNA was digested with *Eco*RI and probed with an *Hind*III fragment from

35   the 5' end of the rosy$^+$ gene in Carnegie 30. This fragment identifies genomic fragments of different sizes according

25

to the position of the nearest *Eco*RI site at the point of
integration of the construct. The common band is from the
· *ry*[506] gene originally present in the host strain.

5   **Figure 4.** Northern analysis of total RNA from fat bodies
     (F) and remaining carcass (C) of third instar larvae of
     each of the four transformed lines (B3 and K2 carry *αlacZ*,
     C1 and D1 carry an *αCYP2B1* insertion). Each track contains
     the RNA from half a larval equivalent. Three identical
10   filters were produced and each probed separately with the
     *CYP2B1 Eco*RI fragment (left), the *lacZ Eco*RI fragment
     containing *Adh*, *lacZ* and SV40 sequence (middle), and a 200bp
     *Bam*HI fragment from the start of the *LSP1α* gene. Z, P, A
     and α denote the *αlacZ*, cytochrome P450, alcohol
15   dehydrogenase and *LSP1α* transcripts respectively.

     Figure 5.   Third instar fat bodies stained for β-
     galactosidase activity or for CYP2B1 protein. (A) *αlacZB3*
     fat body stained blue with X-gal. (B) *αCYP2B1* fat body
20   stained brown showing localisation of the cytochrome P450
     to fat body cells. The salivary glands and testes remained
     colourless.

     Figure 6. Identification of the expressed CYP2B1 protein
25   by Western blotting. (a) Extracts from whole *Drosophila*. C,
     purified CYP2B1 from rat liver microsomes (control); E3,
     early third instar larva; M3, mid third instar larva; L3,
     late third instar larva; P, pupa; A1, 4 h old adult; A2,
     24h old adult; H, mid third instar larva of untransformed
30   host strain. (b) Localisation of the CYP2B1 protein to
     the microsomal fraction of transformed third instar larvae.
     M, microsomes; C, cytosol; P, 0.5pmol of purified CYP2B1
     from rat liver.

35   **Figure 7.** Breeding schemes to introduce the transgenic

26

constructs inserted on chromosome II into the genetic backgrounds for performing the SMART assay.   B'Y: derivative of Y chromosome with dominant B' mutation that causes extreme Bar eyes.   C1B: first chromosome balancer

5   with dominant Bar eye marker, recessive lethal therefore males die.   e: ebony body.   flr³: abnormal trichomes and bristles, recessive zygotic lethal but viable as small clones of homozygous cells.   fs(1)K10:   abnormal egg shape when homozygous in germ line cells.   mwh: multiple wing

10   hair, 2-7 processes instead of 1 per wing blade cell.   se: dark brown eyes to facilitate recognition of eye mosaic spots.   TM2,Ubx se eᵇ: third chromosome balancer with recessive lethal, Ultrabithorax, which causes enlarged halteres.   w:white eye.   wᶜᶜ:coral red eyes.   y: yellow

15   body.   y⁺Y: translocation of y⁺ allele to the Y chromosome.

Figure 8.   A 92mer oligo corresponding to the sequence for the 24 n-terminal amino acids of the *S. cerevisiae* P450 reductase (Yabusaki *et al* 1988) was synthesised such that

20   *Hind*III and *Sal*I sites were created at the ends.   This oligo was made double stranded using a complementary oligo and PCR, digested with *Hind*III and *Sal*I and cloned into the polylinker of pTZ18R to create pTZF1.

25   The variable regions of the hybrid P450 reductase cDNA's were synthesised by PCR using Primer 1 which hybridises at the *Sac*I site within the rat P450 reductase cDNA and either Primer 2, hybridising at the Lys-Arg Trypsin sensitive junction of rat P450 reductase cDNA or Primer 3,

30   hybridising at the n-terminal region of the rat P450 reductase cDNA.   PCR was performed using a Thermal Reactor supplied by Hybaid, Teddington, UK according to manufacturer's instructions using *Taq* polymerase supplied by IBI Ltd, Cambridge, UK.   The sequences of the fragments

35   synthesised by PCR were confirmed by DNA sequencing.   The subsequent products of the PCR were digested with *Sac*I and

*Sal*I and ligated into *Bam*HI/*Sal*I digested pTZF1 along with
the *Bam*HI/*Sac*I fragment of rat P450 reductase cDNA from
pJLF1 (Bligh *et al* 1990) to create pTZF2 and pTZF3. All DNA
sequencing and manipulations were by standard techniques
5   (Maniatis *et al* 1982) except where stated. Abbreviations:
B = *Bam*HI, H = *Hin*dIII, Sc = *Sac*I, Sl = *Sal*I.

Figure 9.

10   a) pFBY5 and pFBY6 were constructed by ligating the *Hin*dIII
P450 reductase encoding fragments from pTZF2 and pTZF3
respectively into the unique *Hin*dIII site of pAAH5 (Ammerer
(1983)) such that the orientation was correct for
expression from the *S. cerevisiae ADH1* promoter. This plasmid
15   has a *LEU2* selectable marker. pAAH5 was provided by Sandra
Jaeger Thompson.
b)   pFBY8 was constructed by removing the *Bam*HI fragment
containing the hybrid P450 reductase cDNA between the *ADH1*
promoter and terminator from pFBY6, and ligating it into
20   the unique *Bam*HI site of pMA56 (Ammerer 1983). This leaves
a unique *Eco*RI site available downstream of a second *ADH1*
promoter into which a P450 cDNA can be cloned. This
plasmid has a *TRP1* selectable marker.
c)   pFBY7 and pFBY9 were constructed by ligating a rat
25   P450IIB1 cDNA (Black *et al* 1989) and a human P450 IIA6 cDNA
(Miles *et al* 1990) respectively into the *Eco*RI site of pFBY8
such that they were expressed from the *ADH1* promoter.
d)   pFBY3 was constructed by ligating a rat P450 reductase
cDNA on a *Hin*dIII fragment into the *Hin*dIII site of pAAH5
30   such that it was expressed from the *ADH1* promoter.
e)   pFBY10 was constructed by ligating an *Eco*RI fragment
containing the human P450IIA1 cDNA into the *Eco*RI site of
pMA56 such that orientation was correct for expression from
the *ADH1* promoter.

Abbreviations: B = *Bam*HI, E = *Eco*RI, H = *Hind*III, Sc = *Sac*I,

S1 = *Sal*I, *ADH1* = Alcohol Dehydrogenase 1.

Key solid bar   = P450 reductase encoding cDNA,
     hatched bar = P450 encoding cDNA

5

Figure 10. Hybrid P450 reductase proteins as encoded by constructs illustrated in Figures 8 and 9.

a)   Complete rat P450 reductase protein encoded by pFBY3.

b)   Hybrid protein encoded on pTZF2 and pFBY5 consisting of
10   the 24 membrane binding amino acids from the endogenous yeast P450 reductase, fused via an Asp residue to the soluble portion of the rat P450 reductase.

c)   Hybrid P450 reductase protein encoded on pTZF3 and pFBY6 consisting of the 24 membrane binding amino acids
15   from the endogenous yeast P450 reductase, fused via an Asp residue to the entire rat P450 reductase protein. This hybrid protein was also encoded on pFBY7, pFBY8 and pFBY9 as it was found to be the most stable of the three proteins.

20

Figure 11.   Panel A: Western blot of microsomes prepared from S150-2B transformed with 1:pAAH5, 2:pFBY3, 3:pFBY5, 4:pFBY6, 5:pFBY8.   Transformations were performed by the Lithium Acetate method (Ito *et al* 1983).   Microsomes were
25   prepared by harvesting 1 litre of cells in late log phase at 5000 rpm in a Sorval Superspeed centrifuge, resuspending cells in 50 mM KPO_4 buffer (pH7.4) containing 30 mM Dithiothreitol, 1.2M sorbitol and 60 units ml^{-1} lyticase and incubating at 30°C until cells were osmotically fragile.
30   Cells were then washed three times in 1.2M sorbitol and then lysed in 0.1M Tris (pH7.5) containing 50 μg ml^{-1} Nα-P-Tosyl-L-lysine chloromethyl ketone, 100 μg ml^{-1} N-Tosyl-L-phenylalanine ketone by brief sonication (1 min X 60W). Debris and mitochondria were removed by centrifugation at
35   10 g for 10 min and the microsomal fraction isolated by centrifugation of the resultant supernatent at 38K for 1h in a Sorvall OTD55B ultracentrifuge.   Pellets were

resuspended in 0.1M KPO$_4$ (pH7.4) buffer containing 20%
glycerol 1 mM EDTA and 0.1% glutathione at a protein
concentration of 10-20 mg ml^{-1}. SDS-PAGE was performed by
the method of Laemmli (1970) and Western blotting performed
5   by the method of Towbin *et al* (1979) using goat anti
reductase IgG (Wolf *et al* 1979) and a peroxidase-conjugated
rabbit anti-goat IgG Vectastain colour detection kit
(Vector Laboratories, Bretton, Peterborough, UK). Tracks
7 and 8 contain 360 ng and 720 ng of purified rat P450
10   reductase as positive control. Arrows indicate proteins of
molecular weights a) 80 807, b) 78 225, c) 73 301. Panel
B: Northern blot of S150-2B transformed with 1:pAAH5,
2:pFBY3, 3:pFBY5, 4:pFBY6, 5:pFBY8 and probed with 745 nt^{32}P
labelled P450 reductase fragment (see Fig 8). RNA was
15   prepared by the method of Schmidt *et al* (1990) and
approximately 20 μg RNA loaded per track. RNA gel
electrophoresis was performed as described by Thomas (1983)
and blotted onto Hybond N membrane (Amersham Int. plc,
Bucks., UK). Prehybridisation, hybridisation and washing
20   were performed as described by Thomas (1980). For
standardisation the same amount of total RNA was hybridised
to a ^{32}P labelled *Eco*RI fragment carrying the *S. cerevisiae* actin
gene supplied by Dr D Jamieson. ^{32}P labelling was performed
as described by Feinberg and Vogelstein (1983).
25

Figure 12. Western blot of microsomal samples prepared
from S150-2B transformed with 1:pMA56, 2:p56/3a, 3:pFBY6,
4:pFBY7, 5:pFBY8, 6:pFBY9, 7:pFBY10 (Figure 2). Lane 8
contains 720 ng of purified rat P450 reductase. Samples
30   were prepared as described in Figure 4 and blots were
prepared, probed and developed as described in Figure 11.
Arrows indicate protein of molecular weights a) 80 807 and
b) 78 225. pMA56 was provided by Ben Hall and is described
by Ammerer (1983) and p56/3a contains a rat P450IIB1 cDNA
35   and is described by Black *et al* (1989).

Figure 13. Panel A: Western blot of microsomal protein (20 µg per track) prepared as described previously (Figure 11) from S150-2B transformed with 2:pMA56, 3:p56/3a, 3:pFBY7, 4:pFBY8 (Figure 2) and probed with rabbit anti rat

5   P450IIB1 antiserum (Black *et al* 1989). Lane 1 contains 50 ng purified rat P450IIB1 protein (Black *et al* 1989). Panel B: Western blot of microsomal protein (40 µg per track) prepared as described previously (Figure 4) from S150-2B transformed with 1:pMA56, 2:pFBY8, 3:pFBY9, 4:pFBY10

10 (Figure 2) and probed with rabbit anti mouse P450IIA1 antiserum (Miles *et al* 1990). Lane 5 contains 7.5 µg human microsomes as a positive control (Miles *et al* 1990).

Both blots were developed using ^{125}I labelled protein A as

15 described by Black *et al* (1989).

Figure 14 is a diagram of vector pMP172 and its construction, in which the multiple cloning site (MCS) includes, in the 5'-3' direction, *Eco*RV, *Kpn*I, *Sst*I, *Eco*RI and

20 *Bam*HI sites. Repeated flanking rare restriction sites (*Not*I, *Nae*I and *Sfi*I) are shown.

Figure 15 shows the bovine keratin VI promoter construct used in Example 6. This uses a 4.8 kb fragment of the 5'

25 end of the gene incorporating the promoter. Because of the rare restriction sites, the entire construct can be removed as one contiguous piece of DNA for generation of the transgenic animals.

30 **Figure 16.** Effect of skin-specific expression of αGST in transgenic (TG) mice on the level of B(a)P- and DMBA-induced DNA adducts in the skin (A) and lungs (B), following the topical application of 1µmol of each compound.

35

Figure 17 shows the functional regions of P450 reductase.

EXAMPLE 1:   TRANSGENIC *DROSOPHILA*:   METABOLISM OF P450 SUBSTRATES

It is the aim of this present study to demonstrate the effectiveness of transgenic *Drosophila* as a powerful approach for studying the metabolic routes of toxins and carcinogens.

The *Drosophila in vivo* system described herein has been found to be one where the genetic background can be controlled, to allow the measurement of a variety of different cytotoxic and genetic endpoints, and to allow assessment of the relative contributions of, and interactions between, different drug metabolizing enzymes.   Finally, the transgenic *Drosophila* system has the important advantage that it allows a number of different genetic endpoints to be measured.

In order to determine the effect of a particular enzyme in determining the susceptibility towards the mutagenic properties of a drug, it is necessary to ensure that the enzyme is present at the time the drug is administered. The *Drosophila* promoter chosen was that of the LSP1α gene. This contains all the sequences necessary to confer high levels of expression in the fat body of the third instar larva (Jowett, 1985; Delaney *et al*, 1987).   The fat body contains the ovary.   The mammalian gene chosen was CYP2B1 (Wolf, 1986; Adesnik and Atchison, 1986; Doehmer *et al*, 1988), a rat gene encoding one of the main phenobarbital-inducible enzymes (P450IIB1 Nebert *et al*, 1987; 1989).   As a control, the same promoter fragment was fused to the *E. coli* lacZ gene.

MATERIALS AND METHODS.

Gene constructs

    The LSP1α promoter was derived from a series of Bal31
5   exonuclease deletions of a larger genomic fragment (Jowett,
1985). Its proximal end was the *ApyI* site immediately
upstream to the first ATG of the protein encoding region.
This was converted to an *EcoRI* site by addition of a linker,
thereby allowing introduction of cDNA sequences flanked by
10   *EcoRI* sites. The CYP2B1 cDNA was derived from a clone
obtained from Dr Milton Adesnik and consisted of 2bp of 5'
untranslated sequence, the entire protein encoding region,
90 bp of 3' sequence joined to 140 bp of SV40 DNA
containing a poly(A) addition signal. This was inserted
15   adjacent to the LSP1α promoter as an *EcoRI* fragment. The
lacZ gene was taken from the plasmid pCa⁴hsneoAUGβ-gal
provided by Dr Vince Pirrotta and Dr Carl Thummel. The
lacZ gene was excised with *SmaI* and *EcoRI*, the *SmaI* site
converted to *EcoRI* by addition of a linker and then the
20   result was inserted downstream of the LSP1α promoter. The
lacZ *EcoRI* fragment so produced had 120bp of sequence from
the start of the Adh gene from *Drosophila* which provided the
ATG start of the protein encoding region fused in frame the
β-galactosidase encoding region. The 3' termination and
25   polyadenylation signal was a fragment of SV40 DNA. Both
the αCYP2B1 and αlacZ constructs were inserted between the
NotI and *SalI* sites of Carnegie 30, in the opposite
orientation to the rosy⁺ gene.

30   P element-mediated transformation.

    The host strain for transformation was LSP1α^F; LSP1β°;
LSP1Γ°LSP2'ry⁵⁰⁶. The transgenes were coinjected with
pPi25.7wc as helper as described previously (Jowett, 1985).
35   The selection of transformants and mapping of inserts was
performed according to scheme in Figure 2. Southern blot

analysis was performed on single flies once they had been
successfully mated, allowing early determination of the
number of insertions in each transformed line. Flies
carrying single insertions on chromosome II were selected
5   and made homozygous.

Nucleic acid extractions and filter hybridisations

DNA was extracted from several flies or from single flies
10   according to the methods described by Jowett (1986). DNA
was digested with restriction enzyme and electrophoresed in
1x TBE buffer (90 mM Tris, 2.5 mM EDTA, 90 mM boric acid).
Size markers were end-labelled fragments of a 1 kb ladder
(BRL). Gels were soaked in 0.25M HCl for 15 min, 0.5M
15   NaOH, 1.5M NACl for 30 min, 1M ammonium acetate for 30 min
and blotted by capillarity in the same buffer for 4h.

RNA was extracted separately from dissected fat bodies and
the remaining carcass as described by Jowett (1985). RNA
20   samples were heated to 65°C for 15 min in 20 mM boric acid,
0.8 mM EDTA, 6% formaldehyde, 60% deionised formamide and
0.05 μg/μl ethidium bromide. 1/10 vol 50% glycerol with
xylene cyanol and bromophenol blue dyes was added prior to
electrophoresis. The size markers were the same end-
25   labelled fragments as for DNA gels but they were first
heated to 100°C in 60% formamide prior to mixing with the
formaldehye and loading dyes. Electrophoresis was through
1% agarose in 20 mM boric acid, 0.8 mM EDTA overnight at
2V/cm with a magnetic stirrer at the anode. The gel was
30   rinsed 2x 5 min in water and soaked 45 min in 1M ammonium
acetate prior to blotting to nitrocellulose in the same
buffer.

Nitrocellulose filters were baked at 80°C for 2 h prior to
35   prehybridisation at 65°C in 3x SCP (20x SCP: 2M NaCl, 0.6M
Na_2HPO_4, 0.02M EDTA) 0.5% sarcosyl, 100μg/ml denatured
herring sperm DNA. Heat denatured random-primed [32]P-

labelled probes were added to the filters and
prehybridisation solution and left overnight at 65°C.
Filters were washed in 2x SCP, 1% SDS at 55°C and then in
3 mM Tris-HCl pH 8.0 at room temperature 2x 30 min.
5 Autoradiography was with Fuji RX film with intensifying
screens at -80°C.

In situ localisation of the transgenic protein products

10 β-galactosidase activity was shown by dissecting third
instar larvae in phosphate buffered saline (PBS: 130 mM
NaCl, 7 mM Na$_2$HPO$_4$, 30 mM NaH$_2$PO$_4$), fixing with 4%
paraformaldehyde in 1xPBS for 30 min and then incubating in
3 mM potassium ferricyanide, 3mM potassium ferrocyanide, 10
15 mM sodium phosphate pH 7.2, 150 mM NaCl, 1 mM MgCl$_2$, 0.2% X-
gal. Fat bodies from the αlacZ transformants developed a
deep blue colour in 5-10 min.

The cytochrome CYP2B1 protein was localised using a rabbit
20 antisera raised against the purified protein from rat
microsomes. Third instar larvae were dissected in 1x PBS
and the tissues fixed with 4% paraformaldehyde in 1xPSB
overnight at 4°C. The tissues were soaked briefly 2 min in
100% methanol and then washed in 75% methanol/fix 10 min,
25 50% methanol/fix 10 min, 25% methanol/fix 10 min, 1xPBS 10
min, and for 10 min in 1x PBT (1xPBS, 0.2% BSA, 0.1% Triton
X-100). They were then blocked in PBT+10% goat serum for
60 min. The rabbit antiserum used was preabsorbed with
dissected larvae of the αlacZ strain which had been fixed
30 and treated as above. The antiserum was diluted 1 in 200
with PBT+5% goat serum and 25 larvae were incubated
overnight at 4°C with gentle agitation in a total volume of
200μl. The preabsorbed antiserum was diluted a further 5x
with PBT+5% goat serum and then incubated with the tissues
35 from αCYP2B1 larvae for 2h. The tissues were rinsed twice
with PBT, washed 3x 20 min with PBT and incubated 30 min in
PBT+5% goat serum. They were then incubated for 60 min

with the second antibody, goat anti-rabbit conjugated with
horseradish peroxidase (Zymed) diluted 1 in 2000 with
PBT+5% goat serum. The tissues were again rinsed twice in
PBT and then 3x 20 min in PBT before incubating 15 min in

5   PBT containing diaminobenzidine at 0.3mg/ml prior to adding
hydrogen peroxide to a final concentration of 0.03%. The
brown colour was alloed to develop for 10-15 min in the
dark before washing once again in PBT.

10   <u>Preparation of microsomes, enzyme assays and Western
blotting</u>

Drosophila microsomes were prepared by homogenising 4g of mid-
third instar larvae in 15 ml 1.15% KCl, 10 mM potassium

15   phosphate pH7.4, 0.1% phenylthiourea. The homogenate was
centrifuged at 1000g for 5 min and the supernatant respun
at 10,000g for 20 min. The remaining supernatant was
respun at 100,000g for 1h. The final supernatant was the
cytosolic fraction and the pellet, after resuspension in

20   homogenisation buffer without phenylthiourea, was the
microsomal fraction.

Fluorometric assays for activities towards the three
resorufin homologues were performed as described by Burke

25   & Mayer (1974). SDS polyacrylamide electrophoresis and
blotting was performed according to the method of Towbin *et
al* (1979).

<u>Construction of the gene fusions</u>

30

The <u>LSP1α</u> promoter consisted of 1090 bp of the genomic
sequence immediately 5' to the start of the protein
encoding region of the <u>LSP1α</u> gene. This was joined to a
full length cDNA of the <u>CYP2B1</u> gene containing 2 bp of

35   untranslated leader sequence, the full protein encoding
region and 90 bp of 3' untranslated region and a 140 bp
fragment containing an SV40 polyadenylation signal (Doehmer

et al, 1988). A similar construct was made with the same promoter fragment fused to the lacZ gene. This had a 120 bp fragment containing the AUG from the Adh gene of *Drosophila* fused to the lacZ gene with a fragment containing an SV40
5   polyadenylation signal sequence 3'. Both constructs were inserted into the P element vector Carnegie 30 (Mismer and Rubin, 1987) in the opposite orientation to the rosy⁺ gene (Figure 1).

10 <u>Analysis of transgenic flies</u>

Injections were into a host strain carrying variants of the four LSP gene and a partial deletion of the rosy gene: LSP1α^F; LSP1β$^°$; LSP1Γ°LSP2'ry^{306}, as described previously
15 (Jowett 1985). Transformants were selected and mapped according to the scheme in Figure 2. Southern blotting identified those transformants which contained single insertions, and two transformants of each construct with insertions on chromosome II were selected for further study
20 (Figure 3). These transgenic strains were homozygous viable and phenotypically normal.

The expression of the transgenes was analysed in a variety of ways. Firstly, since the LSP promoter is tissue and
25 developmentally specific it was expected that the transgenes would be expressed solely in the larval fat body and only appear during the third instar. Mid-third instar larvae from each of the four transgenic strains were dissected and total RNA extracted from the fat body and
30 carcass. Northern analysis of this RNA using the CYP2B1 gene, the lacZ gene construct and a portion of the LSP1α gene as probes confirmed that the lacZ and CYP2B1 transcripts were found in abundance in the fat bodies of the corresponding transgenic strains (Figure 4). The
35 transcript from the αCYP2B1 transgene was of the expected size although the alacZ gene appeared to produce two transcripts, one 100-200 bp larger than the other. Since

the Adh fragment in the _alacZ_ construct contains most of
the proximal Adh promoter, this could be providing an
alternative site for transcription initiation. The alpha
gene fragment identified both the endogenous alpha
5   transcript and the CYP2B1 transcript but not the very much
less abundant _lacZ_ transcript. The transgene transcripts
had only 70bp of homology with the probe and so only weak
hybridisation signals would be expected. Note also that
the _lacZ_ fragment used as probe contained this Adh fragment
10   and therefore cross hybridised with the proximal and distal
Adh transcripts found in the fat body and carcass of mid-
third instar larvae. The presence of translation products
of the transgenes was confirmed by staining the fat bodies
with X-gal for the _alacZ_ transformants and immunologically
15   for the _aCYP2B1_ larvae (Figure 5). Western blots of third
instar larvae also showed that CYP2B1 was expressed only in
the fat body of third instar larvae and that high levels of
immunoreactive protein were detected in the endoplasmic
reticulum containing microsomal fraction (Figure 6). The
20   immunoreactive protein from transformants migrated as a
doublet and it was the upper polypeptide that comigrated
with the purified protein. Since the _LSP1a_ promoter is
very efficient and the amounts of protein are high, this
may reflect incomplete post-translational modification of
25   the protein in the larval fat body or partial degradation.

Larval microsomes from the strains expressing the P450 gene
gave the characteristic carbon monoxide difference spectrum
with a peak at 450nm, whereas microsomes from untransformed
30   larvae gave no detectable P450 peak at the same protein
concentration (data not shown). This suggested that not
only was the protein likely to be biologically active, but
it was also present at very much higher levels than the
endogenous enzymes. In our experiments we were unable to
35   detect endogenous P450 activity in larval microsomes; this
presumably reflected the relatively low level of activity
present in the control strain. In general, it has been

38

found that levels of total cytochrome P450 content are much lower in larvae than in adults and there are differences between strains (Hällström and Grafström, 1981; Zijlstra *et al*, 1984; Hällström *et al*, 1983).

5

In order to determine whether the expressed enzyme retained its normal substrate specificity microsomal preparations were assayed for their activities towards different model cytochrome P450 substrates, namely some resorufin analogues 10 (Burke and Mayer, 1976). These substrates give a distinct pattern of activity with the different P450 isozymes.

Table 1.

Source of enzyme activity of the form of purified cyt. P450.	Relative Activity*				
	C2		C5		Benz
αlacz microsomes	<0.02		<0.02		<0.02
αCYP2B1 microsomes	<0.02	<	0.08	<	1.00
P450IIB1 [a,b]	0.05	<	4.91	<	9.10
P450IIB1 [c]	0.30	<	0.40	<	3.20
P450IIB2 [a]	0.03	<	0.04	<	0.16
P450IIC6 [b]	0.41	>	0.00	<	0.04
P450IIC11 [b]	0.46	>	0.04	<	0.30
P450IA1 [b]	28.0	>>	0.33	<<	6.98
P450IA1 [c]	7.6	>>	0.06	<<	3.3

Table 1. Comparison of the activities towards resorufin analogues of *Drosophila* microsomes from The αlacz strain with those from the αCYP2B1 strain and with previously published data for the purified enzymes.

* Activities are expressed as nmol/min/mg protein for microsomal samples, and as nmol/min/nmol P450 for the purified proteins.

C_2 = ethoxyresorufin; C_5 = pentoxyresorufin; Benz = benzyloxyresorufin

[a] Data from Wolf *et al* (1988). [b] Data from Wolf *et al* (1986). [c] Data from Rodrigues *et al* (1987).

Drosophila expressing αCYP2B1 showed an activity which was higher towards 7-benzyloxyresorufin than 7-pentoxyresorufin, while no activity was detected towards 7-ethoxyresorufin, consistent with the relative activities

5   published previously (Wolf *et al*, 1988; 1986; Rodrigues *et al*, 1987). Microsomes derived from αlacZ transformed larvae gave no detectable activities towards any of the three analogues. Interestingly, these data also demonstrate that the *Drosophila* P450-reductase can couple effectively with the

10 mammalian P450 enzyme. When the same gene, CYP2B1, is expressed in *S. cerevisiae* it couples successfully with the yeast P450-reductase (Black *et al*, 1989).

EXAMPLE 2:   TRANSGENIC *DROSOPHILA*:   GENOTOXICITY OF

15 **CYCLOPHOSPHAMIDE**

Having established that active P450 could be expressed in *Drosophila* larvae, we determined whether the enzyme had a biological effect *in vivo* using the SMART genotoxicity assay.

20 The particular version of this test chosen was that described by Szabad (1986). This involves two complementary strains carrying genetic markers on the X, Y and third chromosomes. These strains are crossed to generate larvae heterozygous for each of the markers (Table

25 2). We have found that choosing the second chromosome for insertions of the transgenes allows them to be most easily introduced into the marker strains. In addition, two different chromosomal insertions for each construct were used so that the cross to generate the heterozygous larvae

30 for the test would generate transheterozygous second chromosome insertions. This was an important feature of the experimental design as it eliminated any recessive effects caused by each particular insertion. Also, in generating the complementary parental strains, the

35 integrity of each chromosome was maintained by ensuring that heterozygous chromosome combinations either involved

41

balancers or were in males (Figure 7). In this way changes
in the genetic background were kept to a minimum. The
materials and methods were as in Example 1.

5   Cyclophosphamide is a carcinogenic anticancer drug which is
activated by cytochrome P450 *in vitro* to mutagenic derivatives
(Nau *et al*, 1982). It is also activated in normal *Drosophila* by
the endogenous enzyme(s) (Graf *et al*, 1983; Clements *et al*,
1984; 1990). However, since the levels of the transgenic
10   product were so high, it was reasonable to expect that the
transgenic P450-expressing strains would be hypersensitive
to the drug. The lack of measurable endogenous P450
activity in larvae illustrates an important advantage of
using an organism evolutionary distant from mammals in
15   which to express the enzyme.

Injection of cyclophosphamide into third instar larvae

Larvae for treatment with cyclophosphamide were generated
20   from by the following crosses:

(A) $\dfrac{\text{fs(1)K10 w}}{\text{B}^S\text{Y}}$ $\dfrac{\alpha\text{lacZ-B3 mwh se e}}{\alpha\text{lacZ-B3 mwh se e}}$ x $\dfrac{\text{y w}^{co}}{\text{y w}^{co}}$ $\dfrac{\alpha\text{lacZ-K2 se flr}^3}{\alpha\text{lacZ-K2 TM2, se}}$

25

(B) $\dfrac{\text{fs(1)K10 w}}{\text{B}^S\text{Y}}$ $\dfrac{\alpha\text{CYP2B1-D1 mwh se e}}{\alpha\text{CYP2B1-D1 mwh se e}}$ x $\dfrac{\text{y w}^{co}}{\text{y w}^{co}}$ $\dfrac{\alpha\text{CYP2B1-C1 se flr}^3}{\alpha\text{CYP2B1-C1 TM2, se}}$

30
αlacZ-B3, αlacZ-K2, αCYP2B1-D1 and αCYP2B1-C1 are
independent single insertions of either the αlacZ or
αCYP2B1 constructs on the second chromosome. The other
symbols are described in Szabad (1986). 4h egg collections
35   were made from the crosses above and the larvae reared
until late third instar. The larvae were washed in Ringer,
dried and etherized for 90sec. The anaesthetised larvae
were injected with a 0.9% NaCl ± 10 mg/ml cyclophosphamide,
using a drawn out glass-capillary connected to an air-

filled 20 ml glass syringe.   Treated larvae were
transferred to vials and allowed to develop into adults
- which were killed and their wings mounted on microscope
slides in Faure's mountant (Ashburner, 1989).   Wings were
5   scored for <u>multiple wing hair (mwh)</u> and <u>flare (flr)</u> clones
according to Graf *et al* (1984).

To ensure that all larvae were exposed to equivalent
amounts they were injected with the drug in aqueous
10 solution and allowed to develop to adults.   Their wings
were then scored for clones of marked cells.   The results
of administering cyclophosphamide to both the <u>αlacZ</u> and
<u>αCYPC2B1</u> transgenic larvae are given in Table 2a.

15

Table 2a.

Strain and treatment	Number of wings	Type and number of spots (frequency in brackets)		
		Small	Large	Total
lacZ (CON)	98	58 (0.59)	21 (0.21)	79 (0.81)
lacZ (CYC)	140	635 (4.54)	50 (0.36)	685 (4.89)
P450 (CON)	102	75 (0.74)	14 (0.14)	89 (0.87)
P450 (CYC)	137	1165 (8.50)	103 (0.75)	1268 (9.26)

Table 2a. SMART assay on the transgenic *Drosophila* larvae injected with cyclophosphamide.

The progeny from crosses A and B (see material and methods for Ex. 1) are referred to as lac2 and P450 respectively. CON and CYC represent injection with saline and cyclophosphamide respectively.

Analysis of the wings for mosaics showed that in both strains cyclophosphamide caused a highly significant increase in the frequency of total clones in the wing:

Table 2b.

TREATMENTS COMPARED	N_c	N_t	n_c	n_t	f_c	f_t	Chi-square
TOTAL WING SPOTS							
lacZ/CON v lacZ/CYC	98	140	79	685	0.81	4.89	298.7**
P450/CON v P450/CYC	102	137	89	1268	0.87	9.89	722.1**
lacZ/CON v P450/CON	98	102	79	89	0.81	0.87	0.189
lacZ/CYC v P450/CYC	140	137	685	1268	4.89	9.26	186.3**
SMALL WING SPOTS							
lacZ/CON v lacZ/CYC	98	140	58	635	0.59	4.54	306.6**
P450/CON v P450/CYC	102	137	75	1165	0.74	8.50	678.6**
lacZ/CON v P450/CON	98	102	58	75	0.59	0.74	1.339
lacZ/CYC v P450/CYC	140	137	635	1165	4.54	8.50	18.82**
LARGE WING SPOTS							
lacZ/CON v lacZ/CYC	98	140	21	50	0.21	0.36	3.479
P450/CON v P450/CYC	102	137	14	103	0.14	0.75	43.86**
lacZ/CON v P450/CON	98	102	21	14	0.21	0.14	1.283
lacZ/CYC v P450/CYC	140	137	50	103	0.36	0.75	18.82**

** Significant at the P << 0.001 level.

- **Table 2b.**   Statistical analysis of the frequencies of
  clones in the two transformed strains. N_c and N_t are the
  number   of   wings   in   the   control   and   test   groups
  respectively. n_c and n_t are the number of spots in the
  control and testwings. f_c and f_t are the control and test
  group frequencies of spots per wing.   Chi-squared was
  calculated as:

$$X^2 = \frac{(|n_c - P_o n| - 0.5)^2}{P_o n} + \frac{(|n_t - q_o n| - 0.5)^2}{q_o n}$$

where $P_o = N_c/(N_c + N_t)$ $q_o = N_t/(N_c + N_t)$ $n = n_c + n_t$

Background frequencies of mosaics in the two strains were
low and no significant difference was found between them.
Comparison of the frequency of mosaic spots between both
strains treated with cyclophosphamide revealed a highly
significant increase in senstivitiy towards the drug for
the αCYP2B1 strain.

These data show that *Drosophila* expressing mammalian
cytochrome P450 enzymes exhibit hypersensitivity towards
cyclophosphamide-induced genotoxic effects.   They also
provide direct evidence that the enzyme metabolizes the
compound to products which are mutagenic *in vivo*.   It is
important to note that the site of metabolic activation was
the fat body whereas the target sites for the genotoxic
effects were the wing imaginal discs.   This clearly
demonstrates the ability of the reactive metabolite to
migrate from one cell type to another.   These studies
illustrate the potential of this system both for testing
the role of specific rodent or human enzymes in the
metabolism and activation/deactivation of chemical toxins
and carcinogens and for studying the *in vivo* properties of
chemical toxins and carcinogens themselves. The ability of
reactive metabolites to migrate from their site of

activation to potential stem cell targets for example is a central issue in chemical carcinogenesis. The markers used in the SMART test allow detection of mosaics in different target tissues: the eye, wing and germline as well as

5   nondisjunction in the male and female progeny of the F2 therefore allowing assessment of different genotoxic effects in different types of tissue (Szabad, 1986). This model can be applied to a wide range of enzymes involved in drug metabolism such as human cytochromes P450, glutathione

10   S-transferases etc, as well as to important enzymes involved in protection against environmental chemicals, for example peroxidases, DNA repair enzymes and even membrane transport proteins such as P-glycoprotein. In addition, by crossing strains carrying different drug metabolizing

15   enzymes, studies can be directed to dissect out the delicate balance between drug activation and detoxification. It will also allow complete metabolic cascades involving drug activation and detoxification pathways to be established. The power of *Drosophila* genetics

20   make this just the starting point for the fine tuning of such metabolic systems.

     Drosophila is used as an alternative to mammals for *in vivo* genotoxicity testing. There are marked differences in the

25   ability of *Drosophila* to metabolize certain xenobiotics such as polycyclic aromatic hydrocarbons when compared with mammalian systems (Zijlstra *et al*, 1987). With the development of gene-targeted P element-induced mutation (Ballinger and Benzer, 1989; Kaiser and Goodwin, 1990), it

30   is now possible to generate *Drosophila* strains in which endogenous genes for xenobiotic-metabolising enzymes have been replaced by human genes, providing a model system which can be tailored to resemble different human drug-metabolising combinations.

35

EXAMPLE 3:   TRANSGENIC YEAST EXPRESSING YEAST/RAT HYBRID
P450 REDUCTASE

Cytochrome P450 enzymes are a super genefamily of
5   haemoproteins involved in a variety of biotransformation
systems and are found in most organisms and in mammals in
multiple forms.   By contrast, the co-enzyme
NADPH:cytochrome P450 reductase is only present in a single
form in mammals and acts as a co-enzyme for all multiple
10 forms of cyt. P450. Mammalian cyts. P450 cDNAs have been
expressed with varying success in *S. cerevisiae* (eg Oeda *et al*
1985, Black *et al* 1989), while attempts to express mammalian
P450 reductase have resulted in very low levels of
expression (Murakami *et al* 1987).   The expression of
15 mammalian cyt. P450 systems in yeast allows study of
individual enzyme metabolism potential and also allows the
study of the interaction between cyt. P450 and P450
reductase. Overexpression of the endogenous *S. cerevisiae* P450
reductase has recently been reported and shown to improve
20 the metabolic activity of mammalian cyts. P450 expressed in
yeast (Murakami *et al* 1990).   However, given that the
identity of mammalian P450 reductases is usually over 90%
and that the identity between rat and *S. cerevisiae* P450
reductases is only 35%, it is highly probable that this
25 improvement in cyt. P450 activity will not always be
possible by solely overexpressing the yeast enzyme.   Here
we report the construction of two yeast-rat P450 reductase
fusions and their expression in *S. cerevisiae*.

30 **Bacterial and yeast strains:** *Escherichia coli* strain NM522 was
used for cloning experiments.   *S. cerevisiae* strain S150-2B
(MATa,his3-Δ1,leu2-3,leu2-112,trp1-289,ura3-52,cir+)   was
used in all expression experiments.

35 **Media and Growth conditions:** Bacteria were cultivated in
Luria-Bertani medium (Miller, 1972).   Yeast transformed

with <u>LEU2</u> selection plasmids were cultivated on minimal
medium containing 0.67% yeast nitrogen base without amino
acids (Difco), 2% glucose, 20μg per ml uracil, 2% agar and
required amino acids (20μg per ml). Yeast transformed with
5   <u>TRP1</u> selection plasmids were cultivated on the same medium
but with a supplement of 1% casamino acids (Difco) instead
of single amino acids. Recombinant yeast strains were
cultivated aerobically at 30°C in the above media without
agar.
10

**Construction of P450 reductase fusion cDNAs and expression
vectors.** The *S. cerevisiae* strong constitutive *ADH1* promoter
was used throughout. This promoter has been successfully
used for expression of mammalian cyts P450 in yeast
15   previously (Oeda *et al* 1985). Hybrid cDNAs were constructed
by adding an oligo encoding the *S. cerevisiae* P450 reductase n-
terminus onto varying lengths of rat P450 reductase cDNA
(Figure 8) and then the entire hybrid cDNA was then
transferred on a single fragment into pAAH5 (Figure 9). A
20   control plasmid, pFBY3, containing the complete rat
reductase cDNA only was also constructed (Figure 9). The
three different proteins produced by the constructs pFBY3,
pFBY5 and pFBY6 and their molecular weights are illustrated
in Figure 10.
25

Expression of hybrid P450 reductase proteins. Expression
of the P450 reductase proteins in yeast was assessed using
Western blotting (Figure 11) and also cytochrome c
reductase activity (Table 3). In both cases it could be
30   seen that the unmodified rat P450 reductase protein (pFBY3)
was not expressed at detectable levels, while both the
fusion proteins (pFBY5 and pFBY6) were produced at levels
detectable by both Western blotting (Figure 11) and
cytochrome c reductase activity (Table 3). Both the fusion
35   proteins produced had the molecular weights predicted from
their DNA sequences (Figures 10 and 11) and RNA of the
correct molecular weight was detectable at similar levels

in transformants of all three constructs indicating that the lack of protein produced by pFBY3 was not caused by the promoter in any way. The lighter of the two P450 reductase fusions appears to be produced in lower quantities than the
5   larger one suggesting that the problems in expressing mammalian P450 reductase in yeast are somehow caused by an unstable protein structure, although other explanations cannot be ruled out. The most stable of the three proteins (produced by pFBY6) contains both *S. cerevisiae* and the rat
10   protein n-termini as well as the soluble moiety of the rat protein, indicating that while the presence of the yeast protein n-terminus contributes significantly to protein stability, possibly by allowing improved targeting into the membrane, the rat protein n-terminus also contributes to
15   the general stability of the protein in some way. It is worth noting that these two n-terminal regions have very little identity compared to other regions of the proteins.

Thus the rat P450 reductase protein expression has been
20   significantly stabilised in yeast by the addition to the protein of the yeast n-terminal 24 amino acids. This stabilisation appears to occur at the protein rather than the RNA level.

25   **EXAMPLE 4:   TRANSGENIC YEAST CO-EXPRESSING HYBRID P450 REDUCTASE AND RAT P450**

As the protein produced by pFBY6 (see Example 3) was the most stable of the three, this fusion was selected for
30   experiments in conjunction with cyt. P450 in yeast and to this end pFBY8 was constructed (Figure 9). This plasmid contains the cDNA for the hybrid P450 reductase protein as well as carrying a second *ADH1* promoter followed by a unique *Eco*RI site for cloning in cyt P450 cDNAs. A second
35   advantage of this construct is that the TRP1 selection enables it to be grown on richer medium (see the Materials and Methods section of Example 3) which appears to improve

50

the yield of protein still further (Figure 11 and Table 3).

- **Table 3.**

PLASMID[a]	CYT.C REDUCTASE[b] ACTIVITY (UNITS)
pAAH5	0.04
pFBY3	0.04
pFBY5	0.09
pFBY6	0.14
pFBY8	0.20

[a] Plasmids and the recombinant proteins produced by expression in yeast strain S150-2B are described in Figures 8, 9 and 10.

[b] Cytochrome c reductase activity was performed on microsomal fractions (see legend to Figure 11). One unit is defined as 1μmole of cyt.c reduced/min/mg microsomal protein. Cyt.c reductase activity was measured using the method of Vermillion and Coon (1978) and protein was quantified using the method of Bradford (1976).

Rat cyt. P450 IIB1 cDNA has previously been expressed on a pMA56-based plasmid (Black *et al* 1989) so this cDNA was initially chosen for study in the pFBY8 based system (Figure 9) using p56/3a as a control plasmid and cloning the cDNA into pFBY8 to creat pFBY7 (Figure 9). While expression of both P450 reductase and cyt. P450 simultaneously appeared to have neither a stabilising or destabilising effect on either protein (Figures 12 and 13), the activity of the cyt. P450 IIB1 as measured by the Benzyloxyresorufin assay was seen to be significantly increased.

EXAMPLE 5:   TRANSGENIC YEAST EXPRESSING HYBRID P450 REDUCTASE AND HUMAN P450

As the identity of human and rat P450 reductases is 93%, it was decided to further extend the expression system of Example 4 to a human cyt. P450 cDNA. Cyt. P450 IIA6 was selected for this (Miles *et al* 1990) and was cloned into pFBY8 to create pFBY9 (Figure 9). As this cDNA had not been previously expressed in yeast, a cyt. P450 only expression vector was also created by cloning the cDNA into pMA56 to make pFBY10 (Figure 9). Expression of this cyt. P450 was confirmed by Western blotting (Figure 13) and P450 reductase levels were also determined (Figure 12 and Table 4). Activity for this cyt. P450, as determined by the coumarin hydroxylase assay (Miles *et al*) was also found to be significantly increased when the P450 reductase fusion was present.

52

Table 4.

PLASMID[a]	CYT.C REDUCTASE[b] ACTIVITY (UNITS)	BENZYLOXYRESORUFIN[c] ACTIVITY pmol/min/mg	COUMARIN HYDROXYLASE[d] ACTIVITY pmol/min/mg
p56/3a	0.04	12	-
pFBY7	0.20	50	-
pFBY8	0.20	nd	0.3
pFBY9	0.20	-	2.1
pFBY10	0.04	-	1.3

[a] Plasmids were as described in Figure 9.

[b] Cyt.c reductase activity was measured as described in Table 3.

[c] Benzyloxyresorufin activity was determined for microsomal samples as described by Black *et al* (1989).

[d] Coumarin hydroxylase activity was determined for microsomal samples as described by Miles *et al* (1990).

Thus, simultaneous expression of the P450 reductase fusion with mammalian cyts. P450 proteins in yeast significantly raises the specific activity of the cyts. P450, without any visible stabilisation of the protein levels. These results indicate that activity of mammalian cyts.

P450 expressed in *S. cerevisiae* is significantly improved when P450 reductase is also overexpressed suggesting that the normal *S. cerevisiae* P450 reductase is deficient at least quantitatively and possibly qualitatively as well. This

5   system will allow the improved expression of other mammalian cyts. P450 in yeast and also enable more detailed study of structural interactions of both cyt. P450 and P450 reductase.

10   **EXAMPLE 6: TRANSGENIC MICE**

With the intention of expressing mammalian (human) proteins (enzymes) in mouse skin, we have constructed a vector which contains the bovine keratin VI promoter. This promoter is

15   homologous to the human keratin 10 and mouse keratin H59, all of which are almost exclusively active in the skin. This enables one to investigate the influence of an expressed enzyme on the toxicity of a chemical applied to a readily accessible and easily observable tissue (skin).

20

The bovine keratin (BK) promoter was kindly donated by José Jorcano but this or similar promoters are described in, for example, Ballieul *et al* (1990) (mouse suprabasal keratin 10 gene), Molt *et al* (1987) (human cytokeratins), Rieger *et al*

25   (1985) (bovine type I cytokeratin), Blessing *et al* (1987) (four bovine type II cytokeratins) and Rothnagel *et al* (mouse K5, K14, K1 and K10 genes). The bovine keratin VI gene is equivalent to the human K10 gene, which is similar to the human K1 gene; the respective promoter from any of these

30   gives skin-specific expression. The bovine keratin 4 promoter (equivalent to human K6) is specific for squamous epithelium, not only skin. Other skin-specific promoters include the tyrosinase promoter. On replacing the promoter's cap-site we cloned it into a vector designed by

35   John Clark (Lathe *et al*, 1987) *Gene* **57**, 193-201), which enables the excision of intact linear inserts for the

production of transgenic mice. The cassette vector
constructed (pMP172) and its method of construction are
· described in Figure 14.

5   Other promoters which are specific for the skin may also be
used in a similar fashion, for example as discussed in
Rothnagel *et al* (1990) *J. Invest. Dermatol.* **94(4)**, 573.

A procedure similar to that described by Murphy and Hanson
10   (1987) (in *DNA Cloning*, Vol III, D M Glover (Ed), IRL Press,
1987), was used to generate transgenic mice which express
the human alpha-class glutathione S-transferase B_1-B_1
isozyme in their skin.

15   Expression was confirmed by Western blotting and
enzymatically by using the alpha-class-specific isomerase
activity of GSTs with androsten-3,17-dione as substrate.

Pilot skin-painting experiments were conducted with
20   heterozygous transgenic mice, investigating the protective
role of GST-B_1B_1 from benz(a)pyrene(B(a)P)- and
dimethylbenzanthracene(DMBA)-induced genetic damage. Using
^{32}P-postlabelling, the levels of DNA-adducts in the skin and
lungs between control and transgenic mice were compared.
25   The results in Figure 16 show that the expressed enzyme
produced a small decrease in B(a)P-induced adducts and a
small increase in adducts by DMBA in the skin. In the
lungs the level of genotoxic damage by DMBA was reduced by
approximately 50% with a small increase in adducts from
30   B(a)P treatment. Any effect in the lungs is not due to
expression of the transgene in that tissue as there is no
detectable level of expression as determined by Western
blot and immunohistochemical analysis. These initial
results show that GST-B_1B_1 may be involved in the
35   biotransformation of DMBA and possibly B(a)P *in vivo*. The
B(a)P result is at least consistent with previous reports,
showing its metabolites to be poor substrates for the

alpha-class GSTs.

Homozygous mice, which express twice the level of protein
in the skin as the heterozygous mice, may be used instead.
5   Other genes which may be expressed in mouse skin and in the
other transgenic hosts of the invention include human
cytochrome P450 enzymes, UDP glucuronosyl transferases,
epoxide hydrolases or any gene involved in xenobiotic
metabolism, DNA repair genes or multidrug resistance genes.
10

The mouse models can then be used to test the involvement
of specific gene expression on the toxicity or
carcinogenicity of compounds of interest. By simple dermal
application of the test chemical, one can then observe the
15   toxic responses on the skin of transgenic versus non-
transgenic mice. This system can be used to establish the
potential risk of industrial chemicals or drugs in
development to man and can also be applied to determine
which chemicals are substrates for the different human or
20   other drug metabolic enzymes expressed in the skin. Such
systems will   also allow pathways important for the
activation or deactivation of chemical carcinogens to be
evaluated.   Other models derived from the original
transgenic lines can be obtained by crossing homozygous
25   lines with mice which: (1) are transgenic for a second
metabolic   enzyme,   allowing   the   investigation   of   a
combination of metabolic pathways in chemical toxicity and
carcinogenicity, and (2) carry mutations or gene deletions
of interest which may make the test more sensitive or
30   provide better analytical end points.

Creation of a Transgenic Mouse

Single cell mouse embryos are harvested from female mice
35   that are impregnated the evening before. The embryos are
treated with hyaluronidase and briefly cultured in M16
medium.   The embryos are transferred to M2 medium on a

56

microscope glass depression slide. The embryos are
observed with a 40X objective and a 10X eyepiece using a
Nikon Diaphot microscope equipped with Hoffman optics. The
embryos are held in place with a holding pipette that has
5   been rounded with a microforge. The positions of both the
holding pipettes and the injection pipettes are controlled
with micromanipulators. DNA as described below is loaded
in the injection pipette at a concentration of 1 to 10
micrograms per milliliter. Approximately one picoliter, as
10   judged by a refractile change of the pronucleus, of DNA
solution is injected into the male pronucleus.

After DNA injection the embryos are transferred to M16
medium and incubated at 37°C in a 5% CO_2 atmosphere for one
15   to two hours. Lysed embryos are discarded and embryos that
appeared normal are transferred to one of the fallopian
tubes of pseudopregnant foster mothers. The transfers are
performed under a dissecting microscope using general
anaesthesia (avertin).
20
After birth, newborn mice are kept with their foster
mothers for 2 weeks, at which point they are then weaned
and screened for DNA integration. A 2 cm portion of the
tail is removed and homogenized in 2 ml of a solution of
25   0.1 M NaCl, 50 mM Tris-HCl, pH 7.5, 1 mM EDTA for short
duration, but long enough to disrupt cell and nuclear
membranes. The homogenized tissue is treated with 50 U/ml
RNaseA and 0.1% SDS for 15 minutes at 37°C. The mixture is
exposed to Proteinase K digestion for 3 hours at 55°C
30   followed by three extractions with phenol/chloroform. DNA
is then precipitated by the addition of ethanol. After
resuspending the precipitated DNA in 10 mM Tris pH 8.0, 0.5
mM EDTA, some of it is digested with BamHI endonuclease and
electrophoresed through an 0.8% agarose gel. The DNA is
35   denatured by soaking the gel in 1.5 M NaCl, 0.5 M NaOH for
one hour and then neutralizing the DNA by soaking it in 1.5
M NaCl, 0.5 M Tris, pH 7.4 for 30 minutes. The gel is then

soaked in 10X SSC for one hour. The DNA is then
transferred from the gel onto a nitrocellulose filter by
the method of Southern, as described in Maniatis, T. *et al.*,
Molecular Cloning, A Laboratory Manual, pp 109-110, 383-389 (Cold
5   Spring Harbor, New York 1982).

The filter with transferred DNA is hybridized overnight
with ^{32}P labelled lambda DNA prepared, according to standard
procedures, by the method of nick translation (Maniatis,
10   *supra*). Following this overnight hybridization, the filter
is washed in 0.1 x SSC, 0.1% SDS at 50°C and Kodak XAR film
is exposed to it in order to identify lambda DNA present
within the mouse genome. Lambda DNA, used as standards,
that has been electrophoresed alongside the mouse genomic
15   DNA is compared in intensity to the transgenic mouse DNA
hybridized to the ^{32}P labelled lambda DNA to estimate copy
number. Numerous transgenic animals may be produced and
identified by this technique and most of them transmit the
integrated DNA to their offspring, demonstrating germ line
20   integration.

The inserted DNA can, theoretically, contain any number or
variety of genes. In the prototype described herein, an *E.
coli* bacteriophage lambda genome has been engineered to carry
25   a β-galactosidase test DNA sequence. The genotype of the
modified lambda genome L2B is lac5 delta (shindill lambda
2-3°) srl lambda 3-5° c1857 xXhl lambda 1° sScll lambda 4°.
Before injecting it into mouse embryos, this lambda DNA is
diluted to a concentration of 10 micrograms per millilitre
30   and the cos ends are annealed and ligated under conditions
predominantly forming circular lambda phage monomers
(Maniatis, *supra*).

Newborn mice are tested for the presence of the test DNA
35   sequence by the tail-blotting procedure (Hogan *et al.*,
Manipulating the Mouse Embryo: A Laboratory Manual pp 174-183, Cold

Spring Harbor Laboratory, 1986). Several of the newborns
are found to carry the test DNA sequence in DNA isolated
from their tails. Eight weeks after birth these transgenic
mice are mated and their progeny are examined for the test
5   DNA sequence. Approximately 50% of the resulting offspring
carried the test DNA sequence, demonstrating that the
original transgenic mice carried the test DNA sequence in
their germ line and that this sequence is inherited
normally. While transgenic lines having approximately one
10 copy of the test DNA sequence per cell can be obtained,
lines having at least about 5-10 copies per cell are
preferred.

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CLAIMS

1.   A transgenic cellular organism useful in an assay for
      determining the metabolism of a compound, the
      organism comprising in the genome of its cell or at
      least one of its cells a coding sequence for
      expressing a polypeptide having the function of a
      naturally-occurring protein which is involved in the
      alteration of the mutagenicity or toxicity of a
      compound under the regulatory control of a suitable
      promoter, the combination of the coding sequence and
      the promoter not normally being found in the said
      cell of the said organism.

2.   An organism according to Claim 1 which is a *Drosophila*
      fly, or a larva or egg thereof.

3.   An organism according to Claim 2 wherein the said
      promoter provides expression in the fat body of the
      third instar larva.

4.   An organism according to Claim 3 wherein the promoter
      is the *Drosophila LSP1α* promoter.

5.   An organism according to Claim 1 wherein the organism
      is a mammal, the said cells which have the said
      coding sequence include skin cells and the said
      promoter is such as to provide expression of the
      coding sequence in the skin cells.

6.   An organism according to Claim 5 wherein the promoter
      is the promoter from a mammalian keratin gene.

7.   An organism according to Claim 5 or 6 which is a
      rodent.

8.   An organism according to any one of Claims 5 to 7
        wherein the cells having the said coding sequence
        also have an expressible coding sequence for a
        polypeptide having the enzymatic activity of a
        (preferably mammalian) glutathione S-transferase.

9.   An organism according to Claim 8 wherein the
        glutathione S-transferase is mammalian.

10.   An organism according to Claim 1 which is a yeast.

11.   An organism according to Claim 10 wherein the said
        polypeptide comprises a region adapted to bind yeast
        membranes such that the polypeptide may be bound to
        such membranes without abolishing the said enzymatic
        activity.

12.   An organism according to Claim 11 wherein the
        membranes are endoplasmic reticulum.

13.   An organism according to Claim 10, 11 or 12 wherein
        the yeast also comprises means to express a
        polypeptide having the enzymatic activity of a non-
        yeast NADPH:cytochrome P450 reductase or means to
        express an increased level of a yeast
        NADPH:cytochrome P450 reductase.

14.   An organism according to any one of the preceding
        claims wherein the polypeptide has the function of
        the mdr transport protein, a glutathione S-
        transferase or an enzyme from the superfamily of P450
        cytochrome dependent enzymes.

15. A fusion protein comprising a first region providing the activity of a naturally-occurring protein from a first organism which protein is involved in the alteration of the mutagenicity or toxicity of a compound and a second region adapted to bind to cell membranes, the second region being homologous to a cell-binding region of a protein in an organism other than the first organism.

16. A fusion protein according to Claim 15 wherein the first organism is a mammal and the said naturally-occurring protein is the mdr transport protein, a glutathione S-transferase, P450 reductase or an enzyme of the superfamily of P450 cytochrome dependent enzymes.

17. A fusion protein according to Claim 15 or 16 wherein the second region is adapted to bind to yeast cell membranes.

18. A fusion protein according to Claim 15, 16 or 17 which comprises the sequence $H_2N-R^1-R^2-R^3-COOH$ wherein R^1 is homologous to the n N-terminal amino acids of *Saccharomyces cerevisiae* P450 reductase where n is 10-30, R^2 is homologous to the n' N-terminal amino acids of human P450 reductase where n' is 10-56, and R^3 provides the enzymatic function of human P450 reductase.

19. A fusion protein comprising a first region having the enzymatic function of a mammalian P450 enzyme and a second region having the enzymatic function of a P450 reductase, preferably a mammalian P450 reductase.

20.   A method of preparing a transgenic cellular organism
        according to any one of Claims 1 to 14, comprising
        introducing into a cell of a cellular organism a
        heritable coding sequence and regulatory sequences
5   therefor adapted to express, in the said cell or in
        progeny thereof, a polypeptide which is heterologous
        to the organism and which is involved in the
        mutagenicity or toxicity of a compound.

10 21.   A method according to Claim 20 wherein the
        polypeptide increases or decreases the mutagenicity
        or toxicity of a compound.

22.   A method of determining the toxicity or mutagenicity
15   of a compound, the method comprising exposing an
        organism according to any one of Claims 1 to 14 to
        the compound and identifying mutations or toxic
        effects.

20 23.   A method according to Claim 22 wherein the method
        also comprises exposing a second organism, which
        second organism is identical to the said organism but
        which lacks the function of the said polypeptide, to
        the compound and comparing the mutations or toxic
25   effects of the compound on the said two organisms.
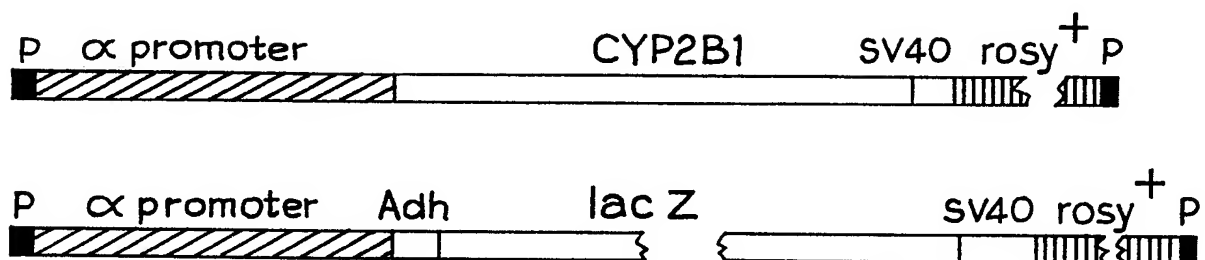
Fig. 1
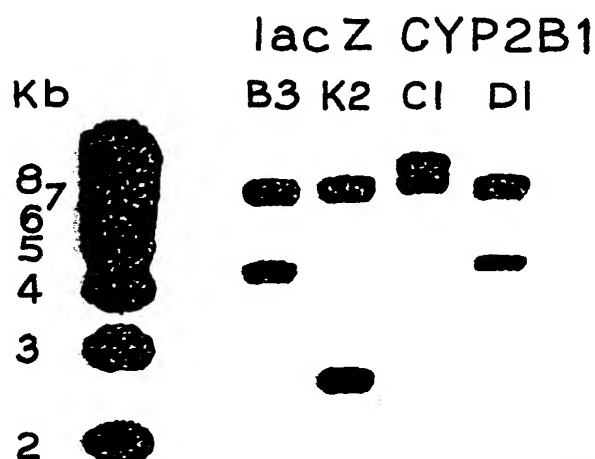
Fig. 3
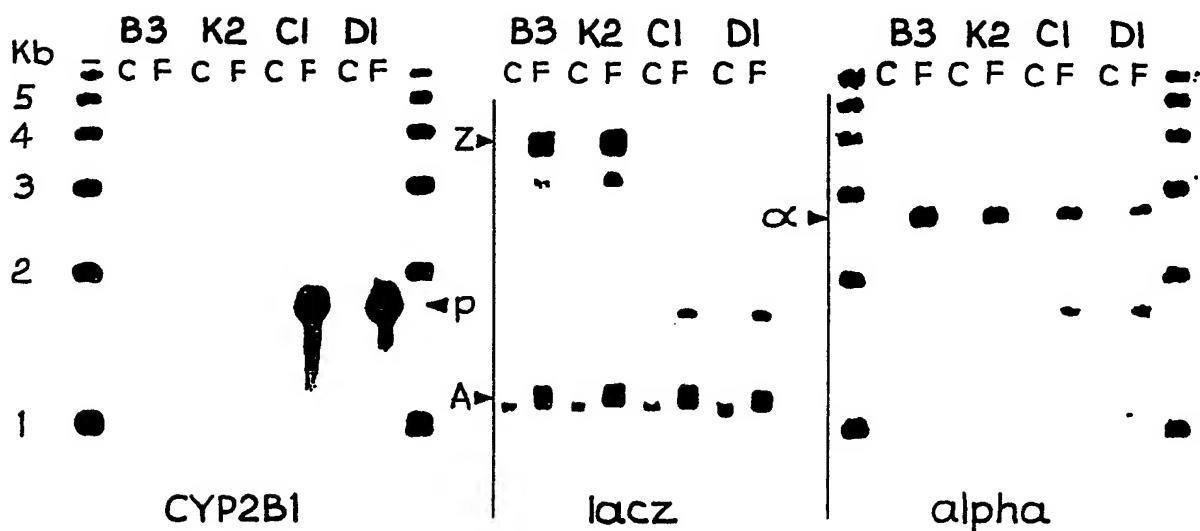
Fig. 4

SELECTION OF TRANSFORMANTS

Host Strain

$$\frac{\alpha^F}{\alpha^F} \; \frac{\beta°}{\beta°} \; \frac{\Gamma° \; 2^S \; ry}{\Gamma° \; 2^S \; ry} \quad x \quad \frac{\alpha^F}{\alpha^F} \; \frac{\beta°}{\beta°} \; \frac{\Gamma° \; 2^S \; ry}{\Gamma° \; 2^S \; ry}$$

Inject embryos with   Ppi25.7wc helper P element
Ppi25.7wc & P[ry$^+$]   to supply transposase
                                         P[ry$^+$] P element construct
                                         carrying ry$^+$ gene and
                                         transgene construct

G0 Mate all surviving adults
   with host strain.

G1 Select transformed ry$^+$ flies x $\quad \frac{\alpha^F}{\alpha^F/Y} \quad \frac{\beta°}{SM1} \quad \frac{\Gamma° \; 2^S \; ry}{TM3, \; Sb \; ry^{RK}}$

After mating extract DNA from
ry$^+$ parents for genomic
Southern analysis.

Select male, ry$^+$, Cy, Sb.
* denotes a possible
P element insertion

G2 $\frac{\alpha^F*}{Y} \quad \frac{\beta°*}{SM1} \quad \frac{\Gamma° \; 2^S \; ry*}{TM3, \; Sb \; ry^{RK}} \quad x \quad \frac{\alpha^F}{\alpha^F} \; \frac{\beta°}{\beta°} \; \frac{\Gamma° \; 2^S \; ry}{\Gamma° \; 2^S \; ry}$

Select male, ry$^+$, Sb

G3 $\frac{\alpha^F}{Y} \quad \frac{\beta° \; P[ry^+]}{\beta°} \quad \frac{\Gamma° \; 2^S \; ry}{TM3, \; Sb \; ry^{RK}} \quad x \quad \frac{\alpha^F}{\alpha^F} \; \frac{\beta°}{SM1} \; \frac{\Gamma° \; 2^S \; ry}{TM3, \; Sb \; ry^{RK}}$

G4 $\frac{\alpha^F}{\alpha^F} \quad \frac{\beta° \; P[ry^+]}{SM1} \quad \frac{\Gamma° \; 2^S \; ry}{TM3, \; Sb \; ry^{RK}} \quad x \quad \frac{\alpha^F}{Y} \; \frac{\beta° \; P[ry^+]}{SM1} \; \frac{\Gamma° \; 2^S \; ry}{TM3, \; Sb \; ry^{RK}}$

G5 $\frac{\alpha^F}{\alpha^F} \quad \frac{\beta° \; P[ry^+]}{\beta° \; P[ry^+]} \quad \frac{\Gamma° \; 2^S \; ry}{TM3, \; Sb \; ry^{RK}} \quad x \quad \frac{\alpha^F}{Y} \; \frac{\beta° \; P[ry^+]}{\beta° \; P[ry^+]} \; \frac{\Gamma° \; 2^S \; ry}{TM3, \; Sb \; ry^{RK}}$

FIGURE 2

A

B

Fig. 5

C   E3 M3 L3 P A1 A3 H   C M P

a   b

Fig. 6

Breeding Scheme A

$$P1 \quad \frac{fs(1)K10\ w}{ClB} \quad \frac{II}{II} \quad \frac{mwh\ se\ e}{mwh\ se\ e} \quad X \quad \frac{\alpha^F}{Y} \quad \frac{\beta^0}{SM1} \quad \frac{\gamma^0\ 2^S\ ry}{TM3,\ Sb\ ry^{RK}}$$

$$F1 \quad \frac{fs(1)K10\ w}{Y} \quad \frac{II}{SM1} \quad \frac{mwh\ se\ e}{TM3,\ Sb\ ry^{RK}} \quad X \quad \frac{\alpha^F}{ClB} \quad \frac{II}{SM1} \quad \frac{mwh\ se\ e}{TM3,\ Sb\ ry^{RK}}$$

$$F2 \quad \frac{\alpha^F}{Y} \quad \frac{\beta^0\ P[ry^+]}{\beta^0\ P[ry^+]} \quad \frac{\gamma^0\ 2^S\ ry}{TM3,\ Sb\ ry^{RK}} \quad X \quad \frac{fs(1)K10\ w}{ClB} \quad \frac{II}{SM1} \quad \frac{mwh\ se\ e}{mwh\ se\ e}$$

$$F3 \quad \frac{fs(1)K10\ w}{Y} \quad \frac{\beta^0\ P[ry^+]}{SM1} \quad \frac{mwh\ se\ e}{TM3,\ Sb\ ry^{RK}} \quad X \quad \frac{\alpha^F}{ClB} \quad \frac{\beta^0\ P[ry^+]}{SM1} \quad \frac{mwh\ se\ e}{TM3,\ Sb\ ry^{RK}}$$

$$F4 \quad \frac{fs(1)K10\ w}{ClB} \quad \frac{\beta^0\ P[ry^+]}{\beta^0\ P[ry^+]} \quad \frac{mwh\ se\ e}{mwh\ se\ e} \quad X \quad \frac{fs(1)K10\ w}{B^SY} \quad \frac{II}{SM1} \quad \frac{mwh\ se\ e}{mwh\ se\ e}$$

$$F5 \quad \frac{fs(1)K10\ w}{ClB} \quad \frac{\beta^0\ P[ry^+]}{SM1} \quad \frac{mwh\ se\ e}{mwh\ se\ e} \quad X \quad \frac{fs(1)K10\ w}{B^SY} \quad \frac{\beta^0\ P[ry^+]}{SM1} \quad \frac{mwh\ se\ e}{mwh\ se\ e}$$

$$F6 \quad \frac{fs(1)K10\ w}{ClB} \quad \frac{\beta^0\ P[ry^+]}{\beta^0\ P[ry^+]} \quad \frac{mwh\ se\ e}{mwh\ se\ e} \quad X \quad \frac{fs(1)K10\ w}{B^SY} \quad \frac{\beta^0\ P[ry^+]}{\beta^0\ P[ry^+]} \quad \frac{mwh\ se\ e}{mwh\ se\ e}$$

$$\frac{fs(1)K10\ w}{B^SY} \quad \frac{II}{II} \quad \frac{mwh\ se\ e}{mwh\ se\ e}$$

FIGURE 7 (START)

Breeding Scheme B

FIGURE 7　(END)

HindIII
TGAAGCTTCATATCATGCCGTTTGGAATAGACAACACCGACTTCACTGTCCTGGCGGGGCTA
         Met

                 SalI   3'
GTGCTTGCCGTGCTACTGTACGTCGACTG
                ValAsp

                       Ligation into HindIII/SalI cut
                       pTZ18R

pTZF1

Primer 1
5'   SacI   3'
    CAGAGCTCATACTGGCGAATGCTCG

+

Primer 2

5' SalI
   CAGTCGACATCCAAACAACGGCCCCACCCGT   3'

                  or

                              Primer 3

PCR and
digestion   5' SalI   3'
                         CAGTCGACATGGGGGACTCTCACGAAGAC

                                    PCR and
                                    digestion
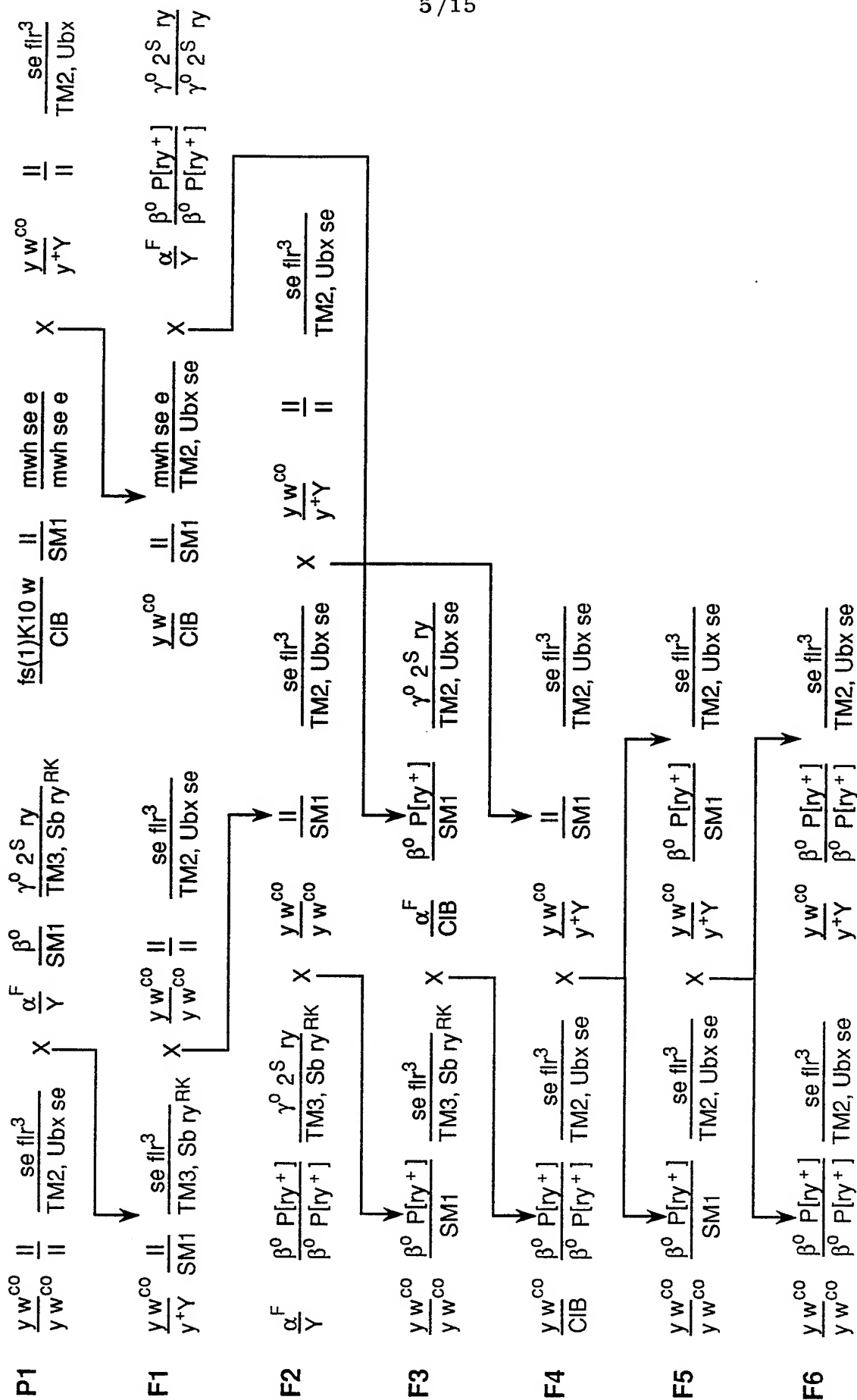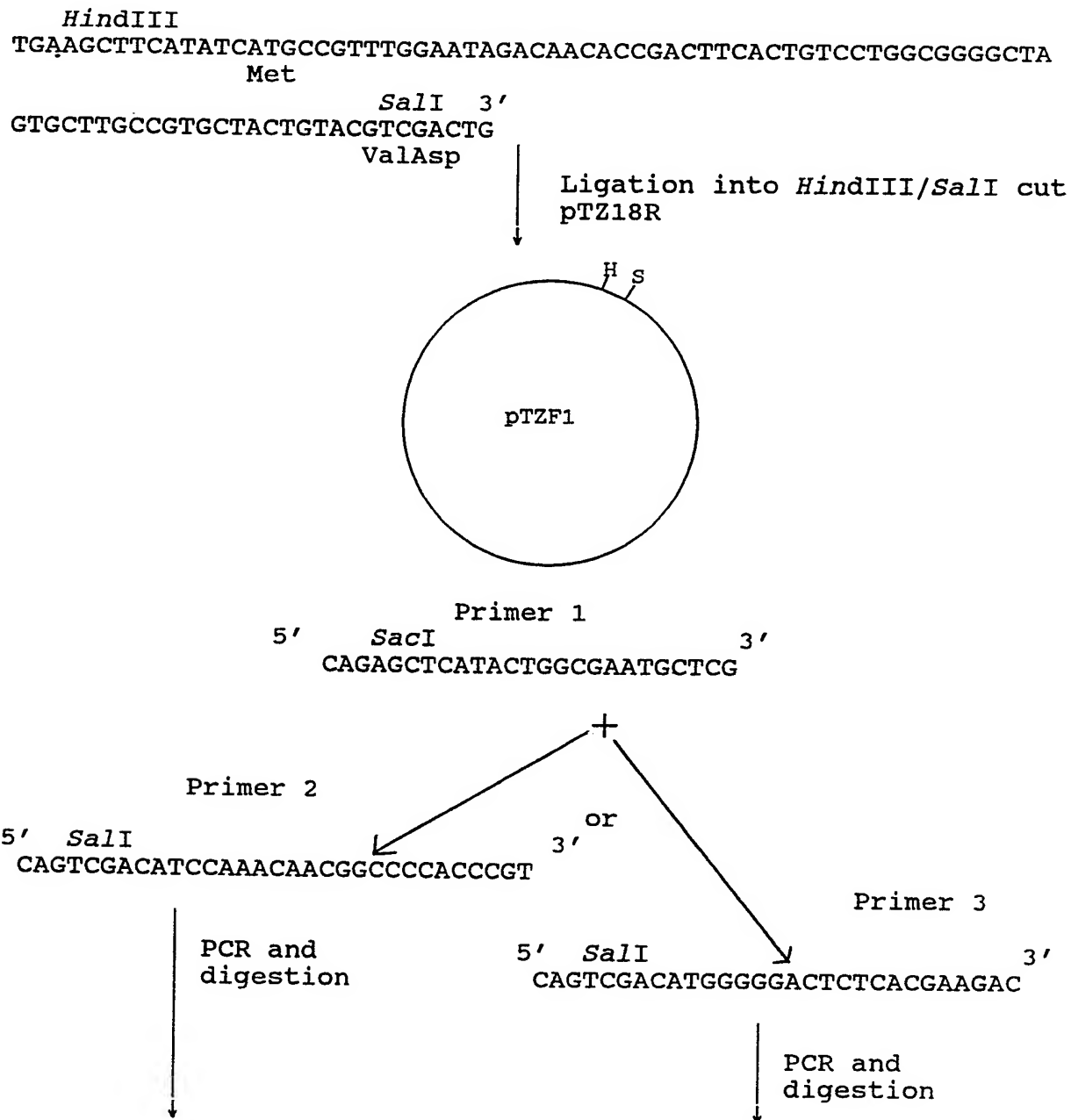
FIGURE 8 [START]

SalI   573nt   SacI   SalI   745nt   SacI

ligation into pTZF1
with SacI/BamHI
fragment of rat
reductase cDNA from
pJLF1
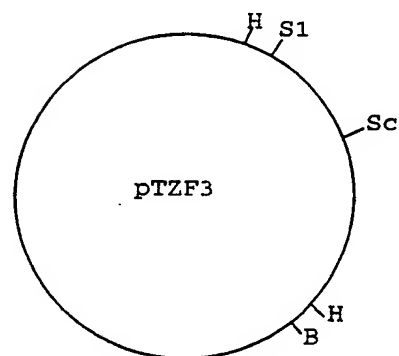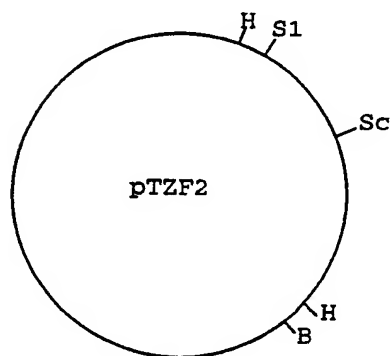
FIGURE 8 [END]

Fig. 9
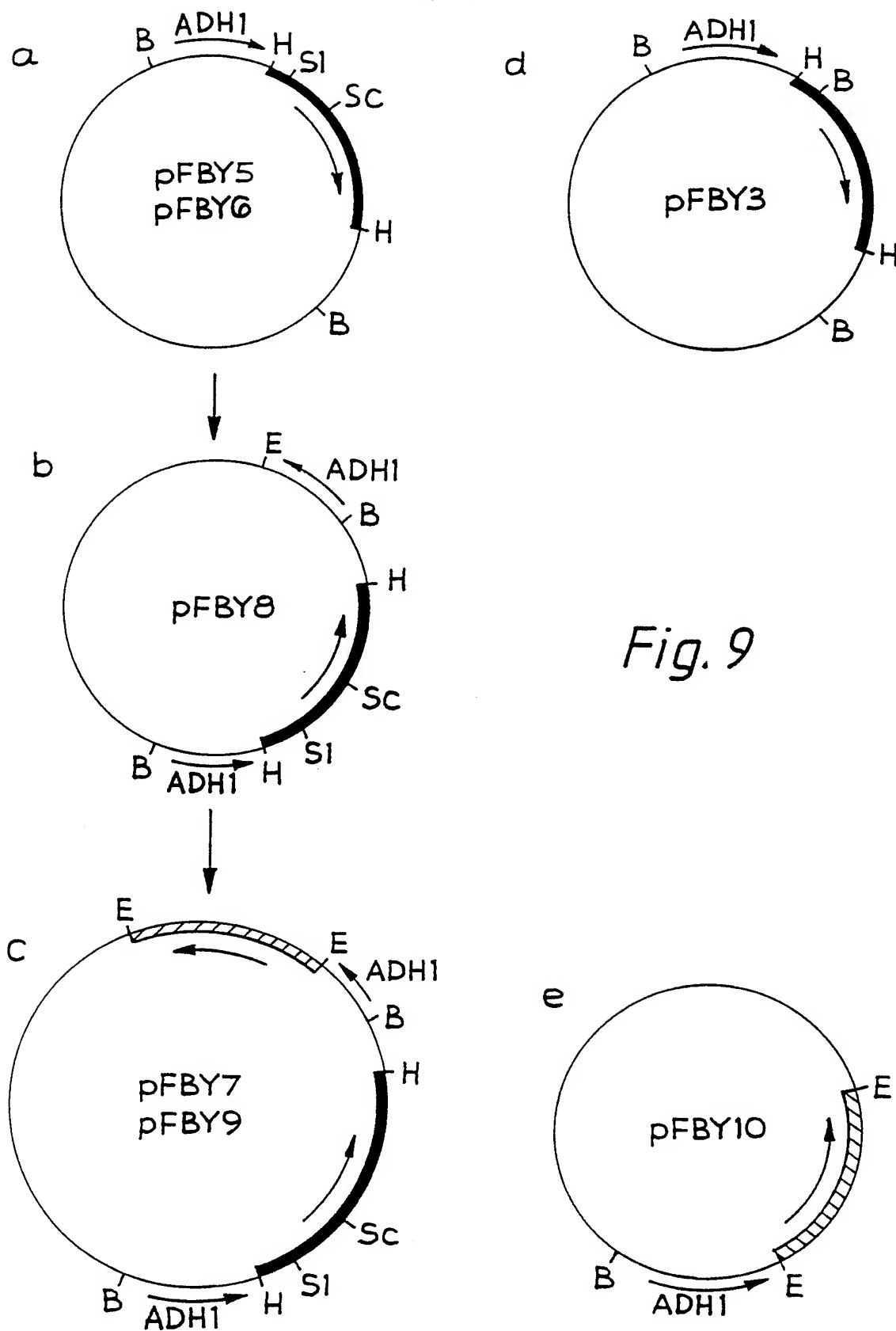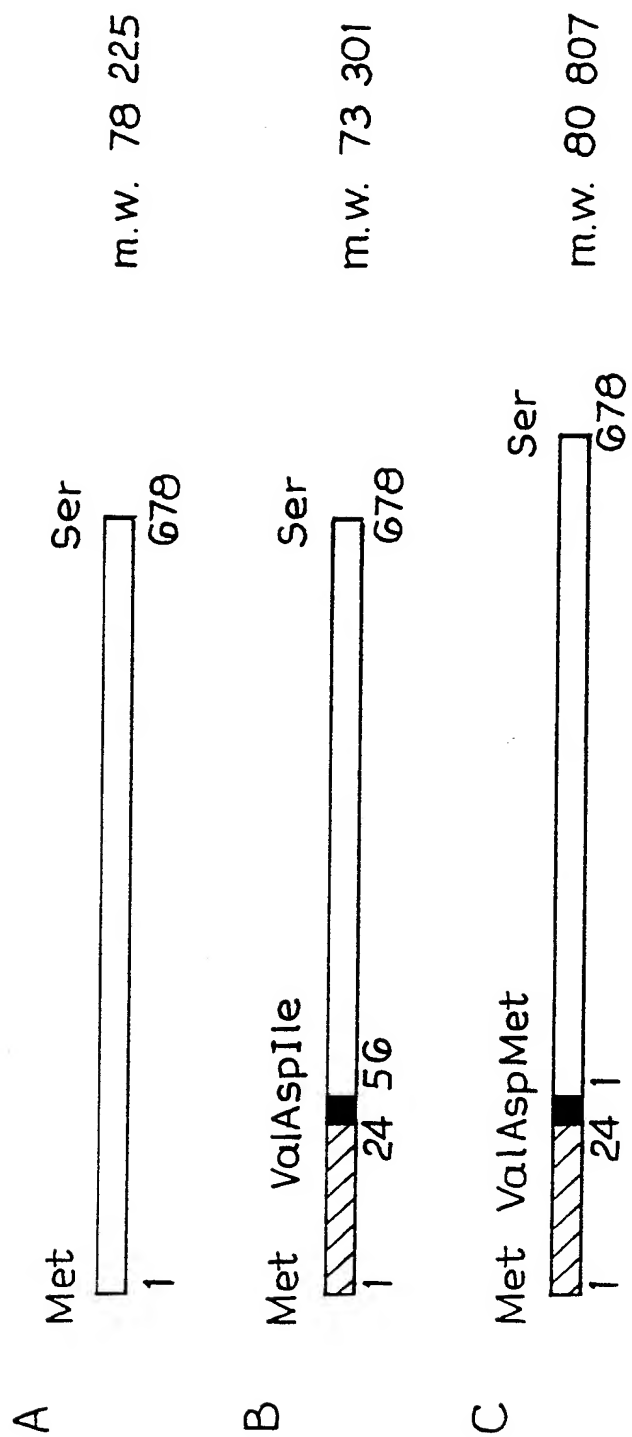
Fig. 10

10/15
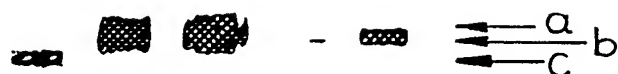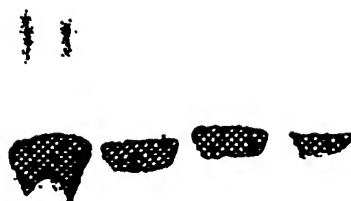
Fig. 11
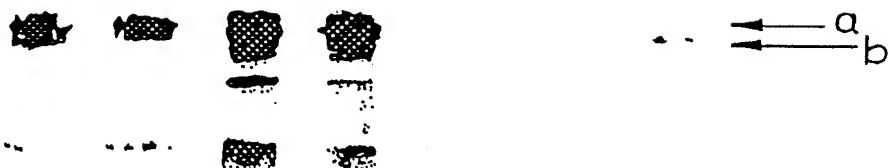
Fig. 12

A   1   2   3   4   5

B   1   2   3   4   5

Fig. 13
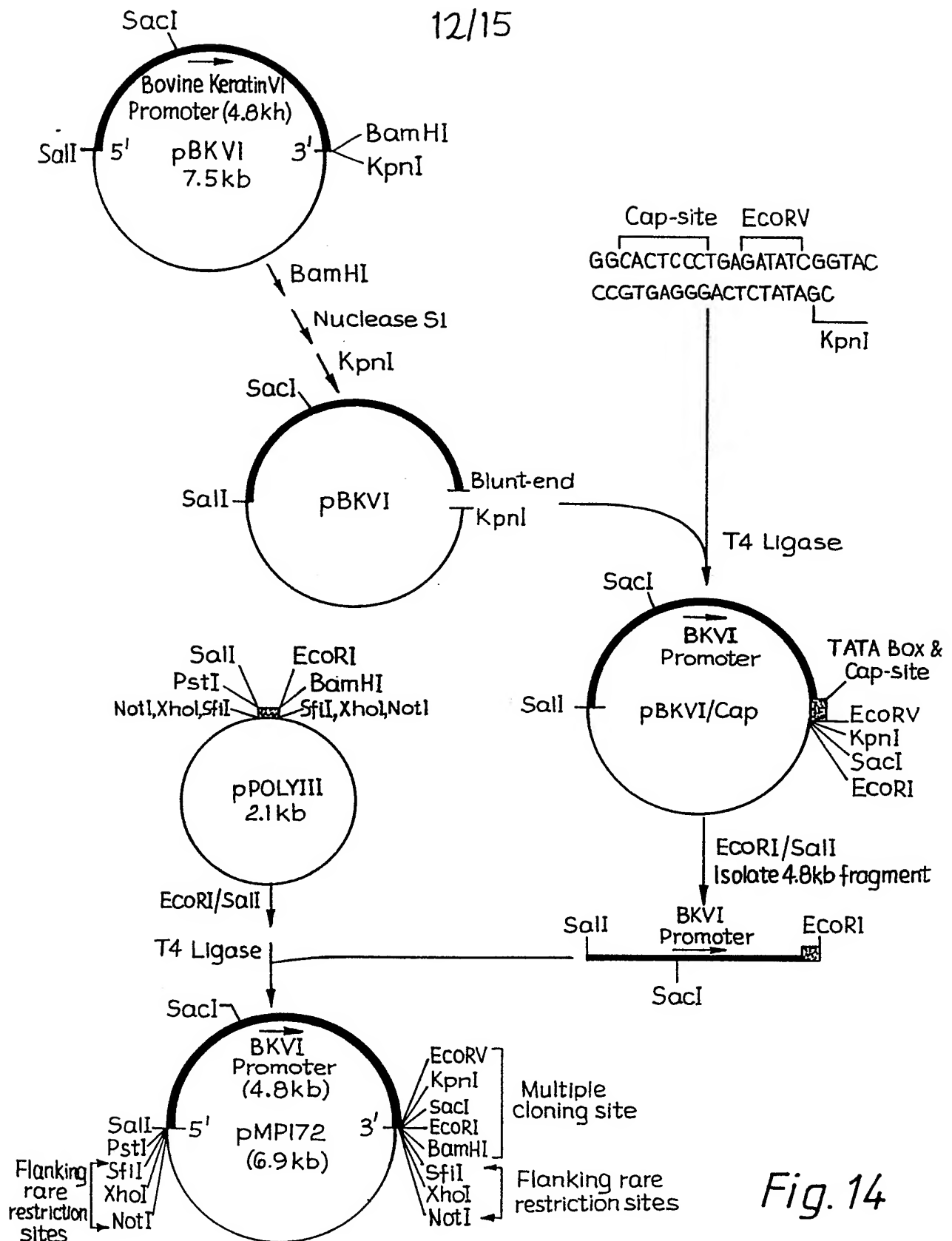
Construction of Bovine Keratin VI expression vector
for skin-specific expression in transgenic mice.

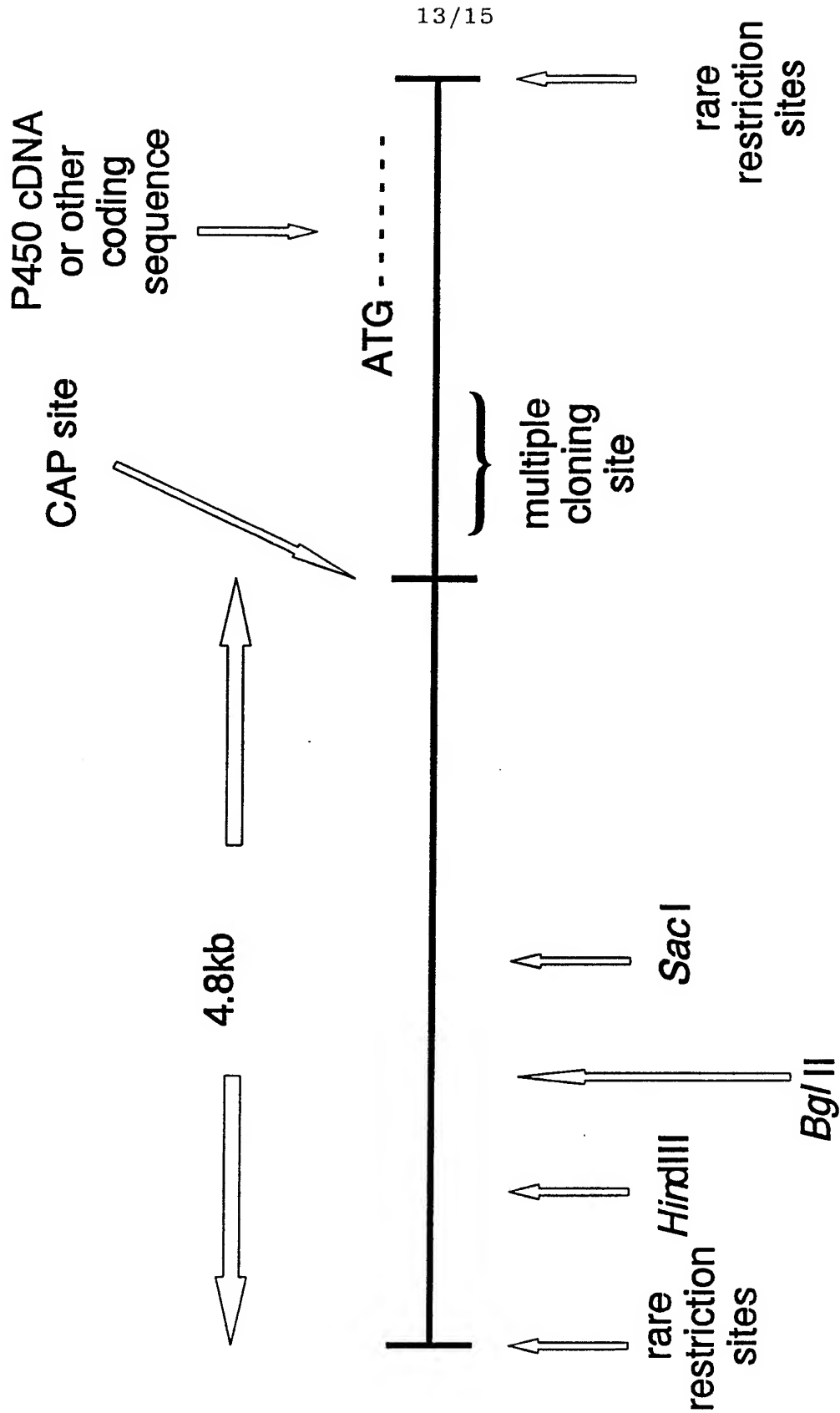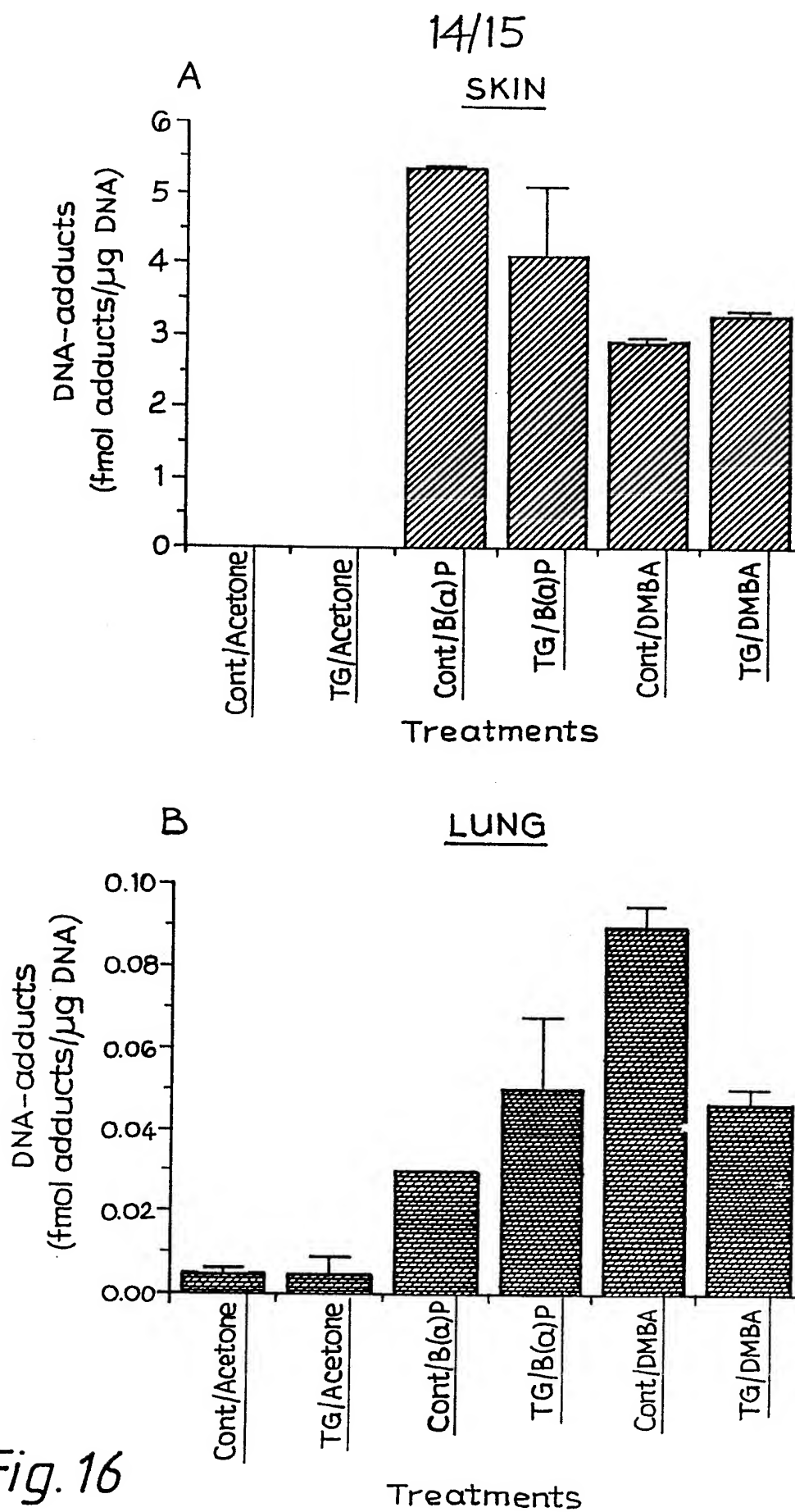Fig. 14

Figure 15
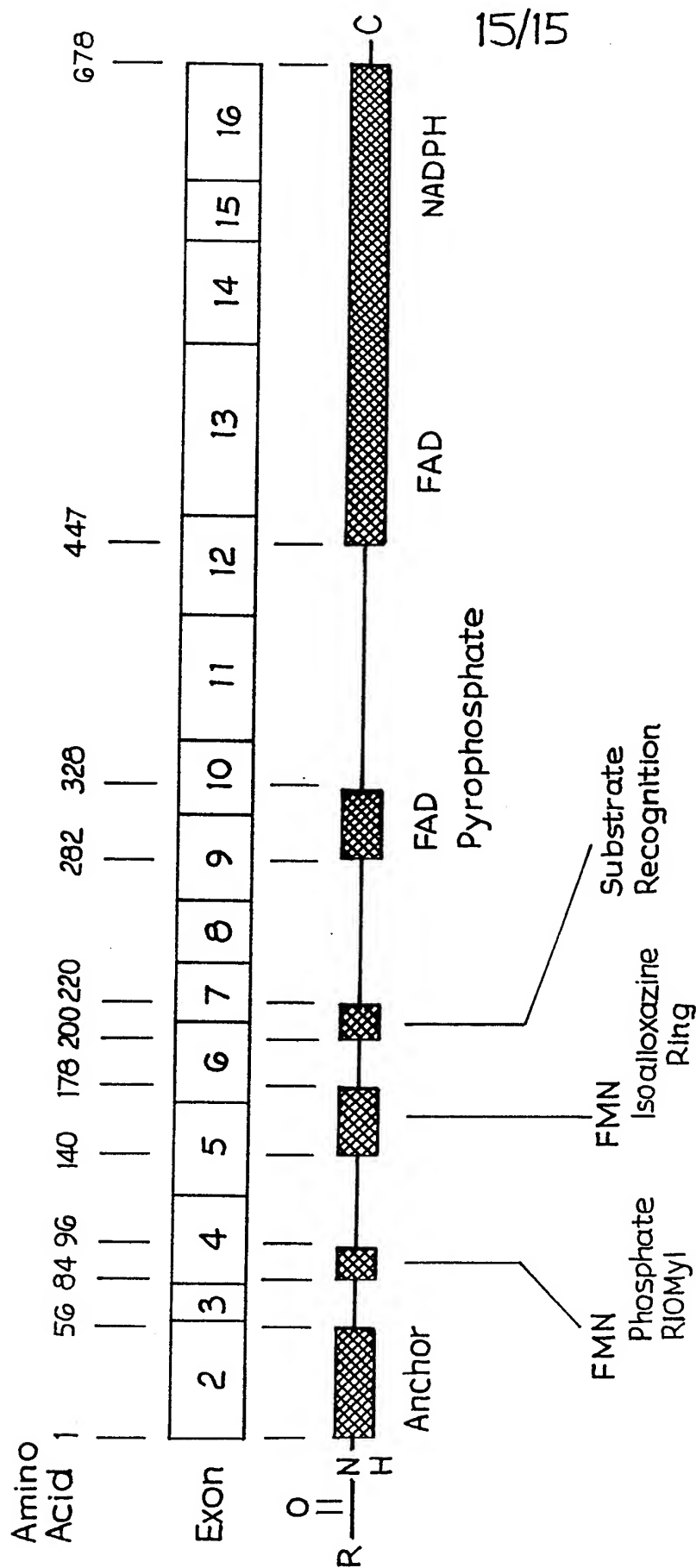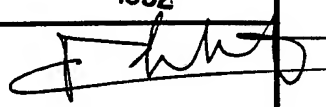
A   <u>SKIN</u>

B   <u>LUNG</u>

Fig.16

Fig. 17

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/00; C12N9/02; C12N15/62; A01K67/027
C12N1/19; C12N9/10; C12N15/12; C12N15/53

II. FIELDS SEARCHED

Minimum Documentation Searched[7]	
Classification System	Classification Symbols
Int.Cl. 5	C12N

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category[°]	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
P,X	EMBO JOURNAL. vol. 10, no. 5, May 1991, EYNSHAM, OXFORD GB pages 1075 - 1081; JOWETT, T ET AL.: 'Mammalian genes expressed in Drodophila : a transgenic model for the study of mechanisms of chemical mutagenesis and metabolism' see the whole document ---	1-4,14, 20,21
X	JOURNAL OF BIOTECHNOLOGY. vol. 9, no. 4, March 1989, AMSTERDAM NL pages 255 - 272; ZURBRIGGEN,B. ET AL.: 'Controlled expression of heterologous cytochrome P450e cDNA in Saccharomyces cerevisiae. I Construction and expression of a complete rat cytochrome P450e cDNA' ---	1,10,14, 20,21

-/--

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
25 MAY 1992	0 9 JUN 1992
International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	CHAMBONNET F.J.

Category°	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.

III. DOCUMENTS CONSIDERED TO BE RELEVANT   (CONTINUED FROM THE SECOND SHEET)

Category°	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 85, no. 16, August 1988, WASHINGTON US pages 5769 - 5773; DOEHMER, J., ET AL.: 'Stable expression of rat cytochrome P-450IIB1 cDNA in Chinese hamster cells (V79) and metabolic activation of aflatoxin B1'	1,20,21, 22
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